University of Alberta

Synthesis of α- and β-Mannopyranoside Epitopes and Preliminary Steps Towards the Identification of β-MannopyranosylTransferases from *Candida albicans*

by

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Abstract

Candida albicans is the most common etiological agent responsible for causing mild superficial vaginal infections in women and invasive life threatening candidemia in immunocompromised patients. The cell wall β-mannan of *C. albicans* is a promising vaccine target as it is highly immunogenic. Monoclonal antibodies raised against the β-mannan confer protection on the host against candidasis.

A cost effective method for the production of the vaccine that avoids multiple synthetic transformations could be based on a chemo-enzymatic strategy using native glycosyltransferases. However, no suitable mannopyranosyltransferases have yet been identified. It is therefore necessary to screen *C. albicans* for the appropriate transferase enzymes. To facilitate this approach, we chemically synthesised a series of oligomannosides through novel and literature protocols. These compounds were then elaborated with a fluorescent moiety (**87-92**) to probe the enzymatic activity of *C. albicans* fungal extracts in the hope of identifying the desired transferases.

α- and β-Mannosidase assays with the products **93**, **94** and **96** from large enzymatic reactions with acceptors **89-91** revealed the presence of only new α-glycosidic linkages. Subsequent NMR experiments confirmed the linkages and the location of the sites of substitution on the glycosylated

hexopyranose residues. β-Mannopyranosyltransferase activity was not observed in any of the experiments performed; therefore, future optimization of the substrates, reaction conditions, and fungal growth conditions needs to be addressed.

Acknowledgements

Some people come into your life only for a short while…
Others traverse your path quickly…
Few walk along side you and leave their imprint on your spirit.

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Some people come into your life only for a short while…
Others traverse your path quickly…
Few walk along side you and leave their imprint on your spirit.

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Some people come into your life only for a short while…
Others traverse your path quickly…
Few walk along side you and leave their imprint on your spirit.

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May God Bless You All, Forever and Always.

Love Magali

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Abbreviations

AA	Ammonium Acetate
Ac	Acetyl
AcOH	Acetic Acid
Ac_2O	Acetic Anhydride
AgOTf	Silver Triflate
AIDS	Acquired ImmunoDeficiency Syndrome
All	Allyl
AllylOH	Allyl Alcohol
APTS	1-Aminopyrene-3,6,8-trisulfonate
Asn	Asparagine
β-1,2-MNT II	β-1,2-Mannopyranosyl Transferase II
B-792	Serotype B Strain of C. albicans
B-Cell	Antibody Producing Cells
BF_3-OEt_2	Boron Trifluoride Ether Complex Bottom of Form
Bn	Benzyl
BnBr	Benzyl Bromide
BSA	Bovine Serum Albumin
n-Bu_4NBH_4	tetra-Butyl-Ammonium Borohydride
n-Bu_4NOAc	tetra-Butyl-Ammonium Acetate
n-Bu_4ONO	tetra-Butyl-
Bz	Benzoyl
C. albicans	*Candida albicans*
^{13}C NMR	1 D-Carbon-13
CAI4	Serotype A Strain of *C. albicans*
CaMNT	*Candida albicans* Mannosyltransferase Gene
CaMNT1	*Candida albicans* Mannosyltransferase I Gene
CaMNT2	*Candida albicans* Mannosyltransferase II Gene
CaMNT1p	*Candida albicans* Mannosyltransferase I
CaMNT2p	*Candida albicans* Mannosyltransferase II
Camnt1-Camnt2	Double Knock-Out Mutant
CCl_3CN	Trichloroacetonitrile
CD_3OD	Deuterated Methanol
CD4	T-Cell Co-Receptor
CD40	B-Cell Tumour Necrosis Factor Receptor
CD40L	Membrane-Bound Effector

CE	Capillary Electrophoresis
CFU	Colony Forming Unit
CH_2Cl_2	Dichloromethane
CH_3CN	Acetonitrile
CH_3NO_2	Nitromethane
CZE	Capillary Zone Electrophoresis
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DMSO	Dimethylsulfoxide
DTBMP	2,6-di-tert-butyl-4-methylpyridine (Base)
DTT	Dithiothreitol
EDTA	EthyleneDiamineTetraacetic Acid
ELISA	Enzyme Linked ImmunoSorbent Assay
EOF	ElectoOsmotic Flow
ER	Endoplasmic Reticulum
ESI HRMS	ElectroSpray ionization High Resolution Mass Spectroscopy
Et_3N	Triethylamine
EtOAC	Ethyl Acetate
EtOH	Ethanol
Fraction P	Resuspended 45k Pellet w/ No Detergent
Fraction POE-P	Resuspended 45k Pellet w/ POE Detergent
Fraction S	Resuspended 45k Supernatant w/ No Detergent
GCOSY	Gradient selected COrrelation SpectroscopY
GHMBC	Gradient selected Heteronuclear Multiple Bond Coherence
GlcNAc	N-Acetyl Glucosamine
GPI	GlycosylPhosphatidyl Inositol anchor
GHSQC	Gradient selected Heteronuclear Single Quantum Correlation
GTOCSY	Gradient selected TOtal Correlated SpectroscopY (HOHAHA)
h	Hour
^1H NMR	1 D-Proton NMR
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	Hydrogen Peroxide
HOHAHA	Homonuclear Hartman-Hahn spectroscopy (TOCSY)
HPLC	High Pressure Liquid Chromatography
HPTLC	High Pressure Thin Layer Chromatography
HSP	Heat Shock Protein
IAD	Intermolecular Aglycon Delivery
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL-4	Interleukin 4
J-1012	Serotype A Strain of *C. albicans*
$^{1}J_{c\text{-}H}$	1-Bond Coupling Constant
$^{2}J_{c\text{-}H}$	Geminal Coupling Constant
KOH	Potassium Hydroxide
K-Selectride	Potassium tri-*sec*-butylborohydride $K[CH(CH_3)CH_2CH_3]_3BH$
LG	Leaving Group
LIF	Laser Induced Fluorescence
L-Selectride	Lithium tri-sec-butylborohydride $Li[CH(CH_3)CH_2CH_3]_3BH$
Mab	Monoclonal Antibody
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time Of Flight
Man	Mannose
MeOH	Methanol
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
MHC	Major Histocompatibility Complex
$MnCl_2$	Manganese Chloride
MT	Migration Time
$NaBH_4$	Sodium Borohydride
NaH	Sodium Hydride
Na/NH_3	Sodium dissolved in Liquid Ammonia (Birch Reduction)
$NaHCO_3$	Sodium Bicarbonate
NaOAC	Sodium Acetate
NaOH	Sodium Hydroxide
NaOME	Sodium Methoxide
Na_2SO_4	Sodium Sulphate
$Na_2S_2SO_3$	Sodium Thiosulphate
NBS	*N*-Bromosuccinimide
NGY24	Homozygous Disrupted CaMNT1 Strain of C. albicans
NIH B-792	Serotype B Strain of C. albicans
NIS	*N*-Iodosuccinimide
NMR	Nuclear Magnetic Resonance
NPOE	N-Pentenyl OrthoEster
ON	Over Night
OTf	Trifluoromethanesulfonate
PBS	Phosphate Buffered Saline

$Pd(OH)_2$	Palladium Hydroxide
Ph	Phenyl
PMB	p-Methoxy Benzyl
POE	octyl-PolyOxyEethylene
RT	Room Temperature
SepPak	Solid Phase Extraction Cartridge
Ser	Serine
$SnCl_2$	Tin (II) Chloride
Swern	Oxidation Conditions with Oxalyl Chlolride (COCl)2
TBABr	tetra-n-Butyl Ammonium Bromide
TBDMSOTf	tert-n-butyl-dimethylsilyltrifluoromethanesulfonate
TBE	Tris-Borate-EDTA
TBES	Tris-Borate-EDTA with 20mM SDS
T-Cell	helper Thymus-Cell
Tf_2O	Trifluromethanesulfonic Anhydride
TfOH	Trifluoromethanesulfonic Acid
Thr	Threonine
TLC	Thin Layer Chromatography
TMSOTf	Trimethylsilyltrifluorosulfonate
Triton-X	Polyethylene Glycol p-tert-Octylphenyl Ether (Detergent)
pTSA	para-Toluene Sulfonic Acid
TT	Tetanus Toxoid
TTBP	2,4,6-tri-tert-butylpyrimidine (Hindered Base)
UV	UltraViolet
YPD	Yeast Peptone Dextrose (media)
Zemplen	Acyl Group Removal with NaOMe

Chapter 1

Carbohydrates and the Chemical and Enzymatic Methods of Glycosylation

1.1 Introduction of Carbohydrates, their Biological Importance and their Classification

Carbohydrates are the most prevalent biomolecules on earth. Emerging from classical carbohydrate chemistry and biochemistry, the study of carbohydrates with respect to structure, biosynthesis, and biology has rapidly evolved into the new discipline of glyobiology.[1] In the post genomic era, glycobiology has gained importance because glycans are secondary gene products that are produced by encoded enzymes. Oligosaccharides are often covalently linked to either proteins or lipids to form glycoproteins and glycolipids, often collectively known as glycoconjugates. The sugar moieties of these hybrid molecules are commonly referred to as glycans. The primary importance of understanding the biological roles of glycans is illustrated in their wide spectrum of functions, ranging from cell-cell adhesion in the inflammatory response[2] to cancer cell metastasis.[3] Furthermore, the significance of carbohydrates is underscored by the growing list of human diseases, such as Tay-Sachs,[4] breast cancer,[5] and muscular dystrophy,[6] that are associated with aberrations in glycan assembly and expression.

Glycoconjugates are commonly divided into three groups. The first distinction is dependant upon the covalent attachment of oligosaccharides to a

protein or a lipid. The second is the further sub classification of glycoproteins into *N*-linked and *O*-linked categories as determined by the nitrogen (N) or oxygen (O) atom involved in the bond between the protein[7] and the terminal sugar of the glycan.[8] Although, three other distinct carbohydrate-peptide bonds have been identified, their rarity will preclude them from the following discussion.[9]

1.1a Carbohydrates in Glycoproteins: *N*- and *O*-Linked

Glycoproteins are found on the extracellular surface of the plasma membrane and in the insoluble extracellular matrix of eukaryotes.[4] They are also secreted into biological fluids such as serum.[4] In addition to their roles in molecular recognition, the oligosaccharide moiety is also crucial in the assistance of nascent protein folding. *N*-linked glycans are attached invariably to the amide nitrogen of asparagine (Asn) side chains through the *N*-acetylglucosamine (GlcNAc) residue of this group's highly conserved pentasaccharide core structure shown below (Figure 1.1).[8]

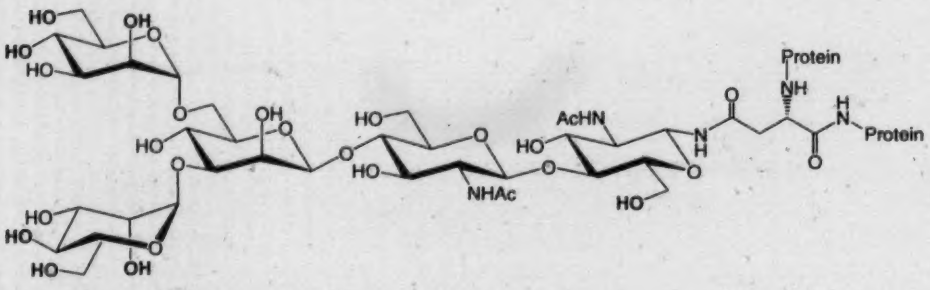


Figure 1.1 Pentasaccharide core structure of all eukaryotic *N*-linked glycoproteins with further modifications possible at the sites highlighted in blue.

Only asparagine residues that are found within the consensus sequence Asn-X-Serine (Ser) /Threonine (Thr), where X may be any amino acid other than proline, can be glycosylated.[10] However, it must be noted that not all asparagine residues found within this sequence of a nascent peptide will be glycosylated. Modification and extension of the *N*-linked glycan occurs in the Golgi apparatus after transfer to its host protein in the endoplasmic reticulum (ER). Glycans are complex in structure and are diverse in the roles they play. For example, the sugar moiety of the glycolipids and glycoproteins on the surface of blood erythrocytes define the ABO and Lewis blood groups.[8,11]

On the other hand, *O*-linked glycans are apparently more randomly situated along the polypeptide because they do not require a specific triplet amino acid consensus sequence for their expression.[8] Different protein-glycan linkages are possible in which mannose, fucose, xylose and *N*-acetylglucosamine may bind to the hydroxyl side chains of serine, threonine, or hydroxylysine residues, however, *N*-acetylgalactosamine is the most commonly observed reducing sugar.[8] Eight core structures have been found in eukaryotes and are dependant upon the nature of the particular glycan.[8,9] The different core structures are characterized by a common GalNAc unit α-linked to Ser or Thr (Figure 1.2).

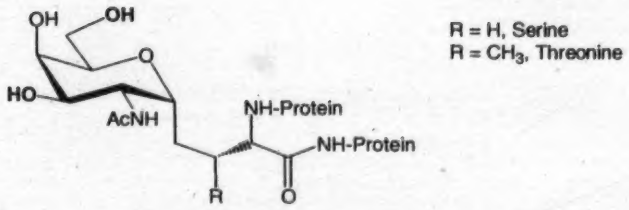


Figure 1.2 Common part of core structure for most *O*-linked glycoproteins with further modifications possible at the sites highlighted in blue.

Despite differences between *N*-linked and *O*-linked glycans, individual glycoproteins can contain both types of glycans as is the case with the plasma membrane protein Glycophorin A.[8]

1.1b Carbohydrates in Glycolipids

Glycolipids make up the third class of glycoconjugates and are embedded in the cellular membrane. The attachment of glycans to specific types of hydrophilic lipid head groups subdivides this class. Glycosphingolipids are able to mimic membrane glycoproteins by presenting potential recognition markers vital to inter-cellular interactions.[8] They also function in the organization of specialized plasma membrane domains and are crucial to the development of the nervous system with respect to myelination. Glycans joined to a ceramide lipid moiety through a β-D-glucopyranosyl unit are known as glycosphingolipids. Addition of a β-D-galactopyranosyl residue completes the lactosyl core and further elongation with *N*-acetylneuraminic

acid residues gives rise to gangliosides, which have been discovered to be tumor associated antigens.[12] However, glycans joined with a phosphatidylglycerol moiety to form glycophospholipids are responsible for anchoring proteins to the surface of the lipid bilayer. Glycophospholipids that are partnered with proteins are more commonly known as glycosylphosphatidyl inositol (GPI) anchors. The glycan links the inositol lipid group to the protein. Again, GPI anchors may be further modified through elaborations of their glycan moiety.[8]

Glycoconjugates have been the focus of increasing research activity during the last 50 years as a result of their extensive biological roles. However, the immense structural diversity and complexity of glycans creates a challenge to the determination of structure and function.[13] Glycobiologists face many challenges in studying glycoconjugates. Carbohydrates have weak affinities and poor selectivity with regards to the proteins that mediate their binding and therefore often require polyvalency to amplify the binding strength and selectivity.[14] Due to the paucity of pure glycan fragments that can be isolated from natural sources, challenging syntheses are often required to produce quantities large enough for studies of biological function. Although progress has been made in the automated synthesis of oligosaccharides by Seeberger and coworkers,[15] a significant obstacle is the lack of general, reproducible solid phase glycosidic bond forming methodologies.[9] Hence, the need to access carbohydrate fragments has spurred advances in

oligosaccharide synthesis through chemical, enzymatic, and chemoenzymatic methods.[16]

1.2 Synthesis of Oligosaccharides

1.2a Introduction to the Glycosidic Bond

The chemical synthesis of oligosaccharides is far more complex than DNA or peptide synthesis due to the need for elaborate hydroxyl group protection strategies and stereospecific glycosylating methods. Glycosylation yields generally fall in the 60 to 80% range, much lower than the usual 99% yield of DNA synthesis.[1] Furthermore, because each glycosylating step cannot be driven to uniform products, the problems of automated synthesis and purification are extreme. The glycosylation reaction is undoubtedly the most important and difficult in carbohydrate chemistry.[17] Novel strategies and concepts such as convergent block synthesis, manipulation of anomeric centre reactivity by armed-disarmed, active-latent, and temporary deactivation have furthered traditional coupling methods. This has opened the way to more efficient synthesis and to previously unavailable carbohydrates.[17] Glycosylation methods have been extensively reviewed and as such only a cursory overview will be presented here.[7,18,19]

The glycosidic bond is formed when the anomeric leaving group (LG) of a glycosyl donor is activated, generally through the use of a Lewis acid, and then nucleophilically displaced by the ring oxygen. The developing oxonium

ion is then trapped by the hydroxyl group of a glycosyl acceptor (Figure 1.3).[17]

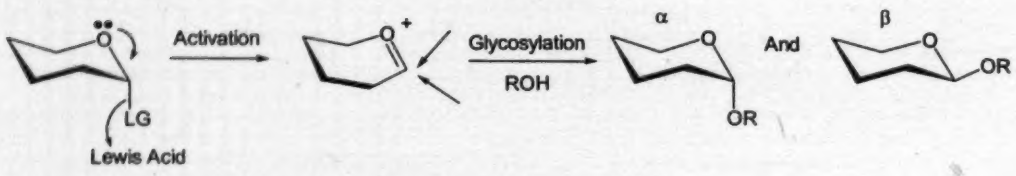


Figure 1.3 Common Mechanism of Glycosylation.

The glycosylation mechanism generally occurs through the formation of an oxacarbenium ion where the resulting anomeric stereochemistry is dependent upon factors such as temperature, pressure, solvent, promoter, steric hindrance, acceptor structure, neighbouring protecting groups[20] and leaving group.[17] Of this list, the participating or non-participating nature of neighbouring groups and the choice of leaving group are the most important factors. Many new glycosyl donors such as thioglycosides,[21] sulfoxides,[22] and trichloroacetimidates[23,24] have been developed over the years since Koenigs and Knorr first introduced the use of glycosyl halides in 1901.[25]

The orientation of the hydroxyl group at C-2 relative to the anomeric substituent is used to classify the type of glycoside bond to be formed as 1,2-*trans*-α, 1,2-*trans*-β, 1,2-*cis*-α, and 1,2-*cis*-β (Figure 1.4). Invariably, 1,2-*cis* linkages are more difficult to form than 1,2-*trans* linkages but the 1,2-*cis*-β is generally the most difficult. The nature of the C-2 protecting

group also heavily impacts and directs the stereochemistry of the glycosidic bond between C-1 and the aglycon.[26]

Figure 1.4 Four most common possible anomeric glycosidic linkages.

1.2b Synthesis of 1,2-*trans*-α and 1,2-*trans*-β Glycosidic Linkages:

Glycosidic linkages in which the C-2 hydroxyl group and the aglycon are *trans* are the easiest to synthesize and were the first to be prepared, and the only ones to be made reliably prior to the 1970's.[27] Both α and β versions can be made under the historically important Koenigs-Knorr reaction conditions utilizing silver carbonate and a reactive nucleophilic alcohol.[25] Although this heterogeneous reaction has been shown to be unimolecular through kinetic experiments, a bimolecular process best describes the resulting overall inverted stereochemistry.[28] It appears that the insoluble silver reagent hinders the bottom face of the molecule and hence forces the alcohol to attack the top face as shown in Figure 1.5.

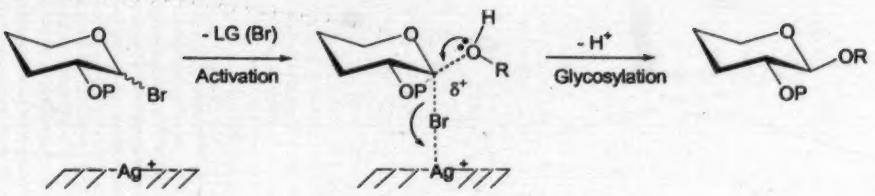


Figure 1.5 Mechanism for the insoluble promoter synthesis of 1,2-β-*trans* linkages.

In the case of the 1,2-*trans*-α linkage in mannose, however, it is well known that the mechanism proceeds through the intramolecular formation of the dioxolenium ion by neighbouring group participation of the acyl protecting group at C-2 (Figure 1.6a). Rearrangement of the orthoester with acid is required to obtain the desired *trans*-glycoside (Figure 1.6b).

(a)

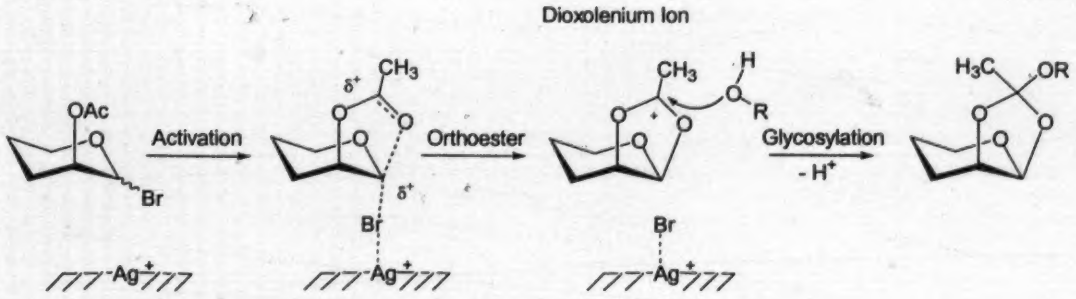


Figure 1.6 (a) Mechanism for the insoluble promoter synthesis of the orthoester intermediate.

(b)

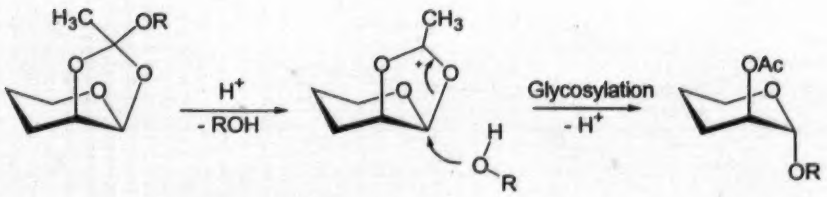


Figure 1.6 (b) Acid-promoted rearrangement of the orthoester to form 1,2-*trans*-α linkages.

Improvements to the Koenigs-Knorr reaction conditions have led to the use of soluble mercuric salts by Helferich, co-solvents and the addition of desiccants.[29,30] Under these conditions, the absence of participating groups at C-2 produces anomeric mixtures with both *cis* and *trans* linkages. It is worth mentioning that reactions carried out in a participating solvent, such as acetonitrile, consistently increase their β-selectivity even in the absence of a participating group.[31] After formation of the oxonium cation, complexation with acetonitrile occurs to form the kinetically controlled α-nitrilium glycosylating species at very low temperatures (Figure 1.7).[32,33] It is inferred that the formation of this intermediate is rate determining and hence the glycosylating step determines the steric outcome of the glycosylation.[31]

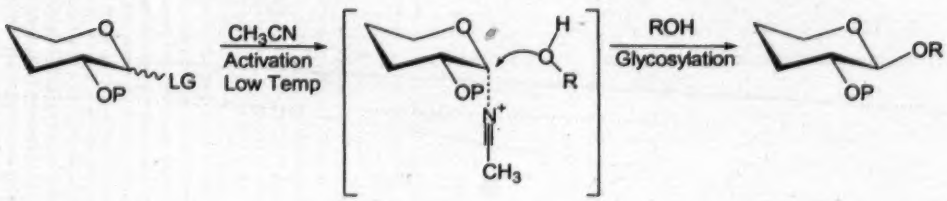


Figure 1.7 Nitrile directed glycosylation. P is a protecting group.

Because of the disadvantages in the intrinsic lability of glycosyl bromides and the need for near equimolar quantities of heavy metal salts in the Koenigs-Knorr and Helferich reactions, other conditions, such as the use of silver triflate and collidine, have been developed.[34] Furthermore, the use of the thioglycoside and trichloroacetimidate methods preclude the formation of orthoester byproducts as their reaction conditions are acidic enough that *in situ* rearrangement occurs. Glycal epoxide donors have also been used to access 1,2-*trans* glycosides.[35]

1.2c Synthesis of 1,2-*cis*-α Glycosidic Linkages:

On the other hand, syntheses of 1,2-*cis*-α glycosides are usually more demanding than their *trans* counterparts, usually rely on the anomeric effect for stereocontrol, and require the use of alkyl non-participating protecting groups on C-2. *Halide Ion Catalysis*, developed in the late 1960's and published in detail in 1975 by Lemieux, was the first reliable synthetic method for forming this linkage.[36] Tetraalkylammonium bromide, a source of soluble halide ions, is used to establish a rapid equilibrium between the stable α-bromide **E** and the much more reactive β-bromide **A** (Figure 1.8a). This more reactive species is then nucleophilically attacked by an alcohol to give the α-glycoside **C** in an S_N2-like fashion. The progression is dependent on the facial availability of various ion-pairs that undergo subtle modifications in the conformation of the different intermediates (Figure 1.8b).[26] A comparison of

the energy of the simplified transition states clearly demonstrates this selectivity (Figure 1.8c).

(a)

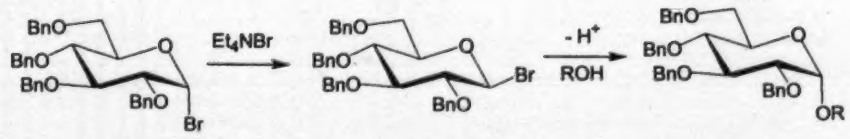


Figure 1.8 (a) Halide ion catalysis of 1,2-*cis*-α glycosides. Taken from R.R. Stick, "Carbohydrates: The Sweet Molecules of Life".[26]

(b)

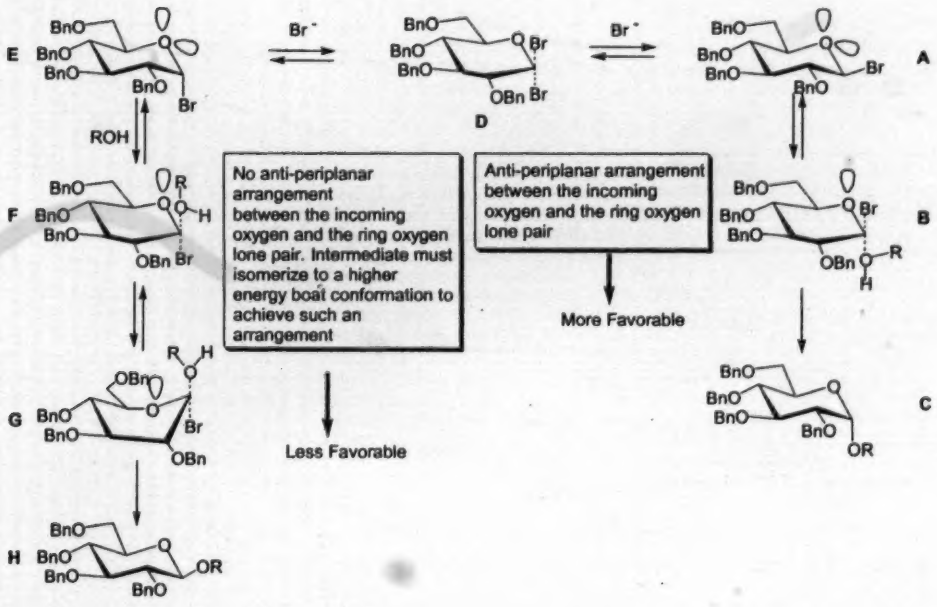


Figure 1.8 (b) Facial availability of various ion-pairs in product selectivity. Taken from T.L. Lowary.[27]

(c)

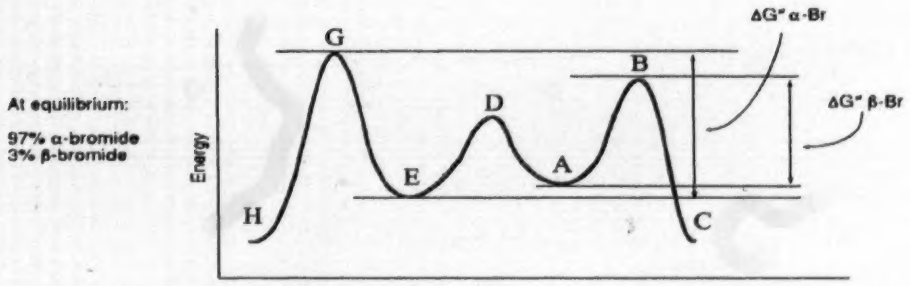


Figure 1.8 (c) Curtin Hammett plot of the product distribution at equilibrium. Taken from T.L. Lowary. [27]

Modern extensions of the *Halide Ion* methodology use the activation of thioglycosides by AgOTf in the presence of NIS to prepare the glycosyl triflate[37] and the activation of trichloroacetimidates with TMSOTf.[23] The observed stereoselectivity is rationalized in the same manner as the *Halide Ion* method.

1.2d Synthesis of 1,2-*cis*-β Glycosidic Linkages:

An efficient and stereoselective synthesis of 1,2-*cis*-β glycosides, such as β-mannopyranosides, remains a formidable challenge despite the development of several novel strategies. This linkage is the most difficult to prepare as the steric and polar effects of the axial C-2 group hinders access to the β-face.[38] Furthermore, adverse neighbouring group participation and the anomeric effect oppose the construction of β-D-mannopyranosidic linkages. The synthesis of these has recently been extensively reviewed; however, a brief overview will be presented in the following section.[39]

Insoluble silver catalysts, such as silver(I) silicate, were traditionally used to prepare β-mannopyranosides through a Koenigs-Knorr like approach with increased S_N2 character.[40] The donor must be suitably activated with alkyl groups and the acceptor nucleophilic enough to trap the oxocarbenium ion before the bromide ion is completely dissociated in order to prevent anomeric product mixtures.[27] Substrate sensitivity and unpredictable yields with α-bromides have led to the development of other approaches.

1.3 Approaches and Advances in the Formation of 1,2- *cis*-β Glycosides

During the last four decades, indirect and direct routes have been established for the construction of β-mannopyranosides. Indirect routes require the epimerization of C-2 of an easily obtained 1,2-*trans*-β glucopyranoside to form the 1,2-*cis*-β glycopyranoside after glycosylation. Direct methods employ an α-mannopyranosyl glycosyl donor and reaction conditions that favour β-glycosylation.

1.3a Indirect Methods

Epimerization of the stereochemistry at C-2 is achieved *via* oxidation-reduction and also by inversion methods.

An oxidation-reduction sequence was first proposed in 1972.[41] Differential hydroxyl protecting groups were used to facilitate the selective oxidation of the 2-OH to form the protected β-D-arabino-hexopyranose-2-

ulosyl-bromide with DMSO in Ac_2O (Figure 1.9 - Path B).[42] Subsequent stereoselective reduction of the ketone with $NaBH_4$ in the presence of "activated" DMSO (acetoxydimethylsulfonium acetate) has been achieved to form the axial alcohol. The use of tetra-*n*-butylammonium borohydride (*n*-Bu_4NBH_4) in tetrahydrofuran has led to an increase in the yield of the manno-epimer.[43] Recently, sterically demanding L- and K-Selectrides have greatly increased the mannopyranosyl product selectivity and yield while decreasing reaction times.[44]

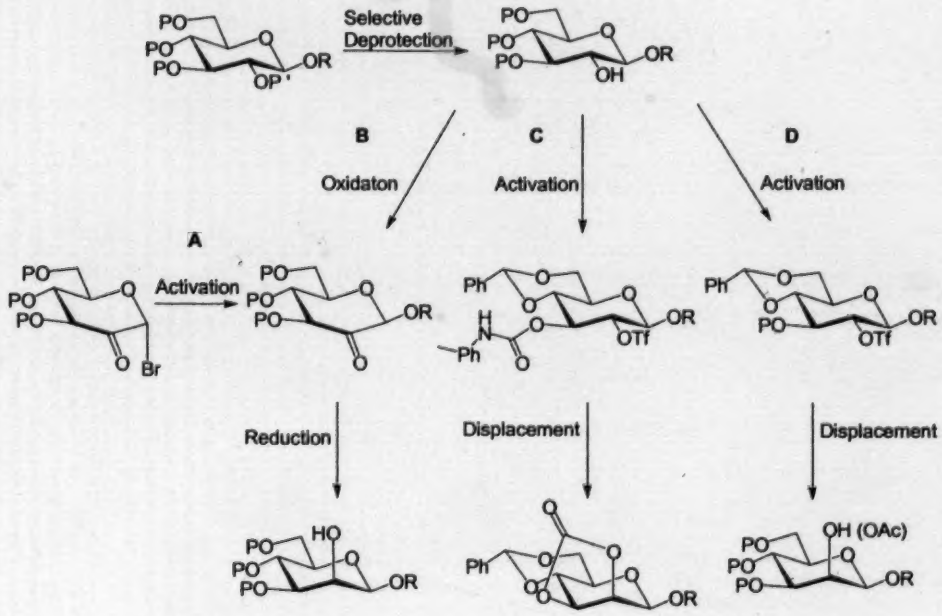


Figure 1.9 Indirect methods of 1,2-*cis*-β glycosides by C-2 epimerization.

Lichtenthaler and Schneider-Adams have extended the oxidation-reduction methodology by their introduction of the 3,4,6,-tri-*O*-benzyl-α-D-arabino-hexopyranose-2-ulosyl-bromide (Figure 1.9 – Path A), which is readily accessible from acetobromoglucose in a four-step sequence.[45,46] Elimination of the deprotection and oxidation sequence after formation of the glycosidic bond streamlines this approach. The heterogeneous silver-aluminosilicate catalyst, used in this strategy, efficiently blocks the axial face of the α-ulosyl bromide donor in a Koenigs-Knorr manner but at the same time suffers from low reactivity. Hence the glycosylation proceeds with exclusive β-selectivity. Facially selective reduction with $NaBH_4$ yields the desired mannopyranoside.

The required C-2 stereochemistry can also be obtained through intra- and inter-molecular S_N2 nucleophilic displacements of 2-*O*-sulfonates (Figure 1.9). Substitutions at C-2 are generally disfavoured, slow and in some instances are further impeded by competing ring contraction reactions that occur when a good leaving group is present at C-2. Introduction of a 4,6-*O*-benzylidene protecting group prevents this side reaction from occurring by stabilizing the pyranose ring in these cases.[47,48] Günther and Kunz have epimerized C-2 by neighbouring group participation using an internal phenylcarbamoyl nucleophilic protecting group at O-3 to displace the O-2 triflate (Figure 1.9 – Path C).[47] Formation of the imino-carbonate intermediate and subsequent mild acid hydrolysis gives high yields of the 2,3-*O*-carbonyl-β-D-mannopyranosides. However, the extensive protecting group

manipulations required in the synthesis to produce the carbamate glucosyl reactant reduces the applicability of this method. Recently, ultrasound promoted nucleophilic inversions of trifluoromethane sulfonates by external terabutylammonium or cesium salts of the desired nucleophile have been quite successful (Figure 1.9 – Path D).[49,50] Furthermore, Fürstner and co-workers have efficiently avoided the 4,6-*O*-benzylidene protecting group and been very successful with the *n*-Bu_4NOAc salt.[49]

1.3b Direct Methods

Although success in the formation of β-mannopyranosidic bonds has been achieved with indirect methods, extensive protecting group manipulations and lack of generality have provided the impetus to finding more 'direct' methods.

Anomeric radical inversion was first introduced in 1988 (Figure 1.10a).[51] Building on the knowledge that anomeric radicals prefer to be axially oriented, to maximize the overlap with a ring oxygen *p*-orbital (Figure 1.10b), Kahne showed that a β-glycoside was generated if an anomeric alkoxy substituent was present.[51] However, only a simple methyl mannopyranoside was formed. Further extension by Crich failed to provide an efficient method with secondary glycosyl acceptors to form β-mannopyranosides.[52]

(a)

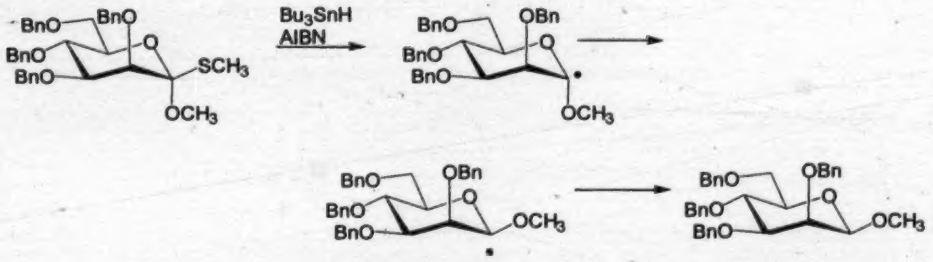


Figure 1.10 (a) Radical inversion of the anomeric center in forming 1,2-*cis*-β glycosides. Taken form D. Kahne *et al.*[51]

(b)

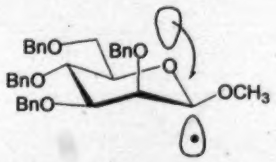


Figure 1.10 (b) Maximal radical stabilization with oxygen *p*-orbital overlap. Taken form D. Kahne *et al.*[51]

Recent advances in direct methods were spearheaded by Baressi and Hindsgaul in their development of the *Intramolecular Aglycon Delivery* (IAD) approach (Figure 1.11).[53] Fundamentally, this method makes use of a tethered alcohol acceptor that captures the developing oxocarbenium ion. An intermolecular acetal between the O-2 of the latent mannosyl donor and the free hydroxyl group of the aglyconic alcohol is first formed. This is followed by concerted intramolecular and stereospecific glycosylation with the activated donor and subsequent quenching with water to yield the desired β-mannopyranoside.

Hindsgaul: Y = C, Z = W = CH_3

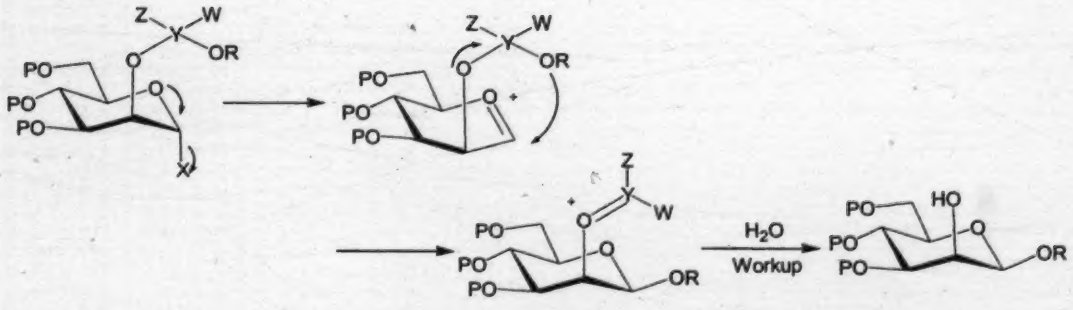


Figure 1.11 Intramolecular Aglycon Delivery. Taken from T.L. Lowary. [27]

Although this first attempt suffered from low yields, this method's feasibility encouraged the extension and development of IAD as exemplified by Stork,[54] Ito,[55] Fairbanks,[56] and Ziegler.[57] This approach has also been the subject of recent reviews.[58]

Stork replaced the carbon atom with silicon in the tethering acetal and took advantage of Kahne's deactivated-activated thiophenylglycoside-sulfoxide leaving group strategy to construct the 6-*O*-D-mannopyranosyl-β-D-glucopyranoside in acceptable yield.[54]

Stork: $Y = Si$, $Z = W = CH_3$

Ito and Ogawa proposed the use of a much more stable methoxybenzylidene tether and radical activation with DDQ under anhydrous conditions in the presence of the desired alcohol to form the acetal.[55]

Activation of a fluoride leaving group with AgOTf and $SnCl_2$ selectively produced the 6-*O*-D-mannopyranosyl-β-D-glucopyranoside in 74% yield.

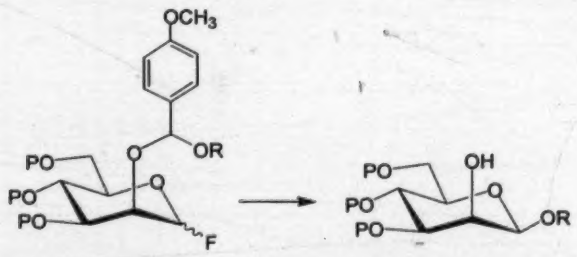


Figure 1.12 Ito and Ogawa's approach to IAD using a methoxybenzylidene acetal. Taken from Y. Ito and T. Ogawa.[55]

Fairbanks offered an increase in tethering efficiency compared to Hindsgaul's method using 2-*O*-vinyl glycosyl donors.[56] The vinyl ethers were acquired through a transvinylation process of alcohols using a vinyl acetate iridium complex as reported by Ishii.[59] The mixed acetals were consequently formed with iodine in the presence of AgOTf and DTBMP. Although optimization of the subsequent intramolecular glycosylation step proved to be difficult, the same reaction conditions for acetal formation were found to be effective, however, only in CH_3CN. Moderate to good yields were obtained in the completely stereoselective formation of a variety of 1,2-*cis*-β glycosides. 1,2-*cis*-α glycosides were also obtained in this manner.[56]

Fairbanks: Y = C, Z =CH_2I, W = H

Ziegler's approach to IAD may be more correctly sub-classified as a "molecular clamp" concept (Figure 1.13).[19] The carbohydrate moieties are joined together via a succinyl bridge between the O-2 of the glycosyl donor and the O-4 of the acceptor.[57] Activation of the thiophenyl donor with NIS and TMSOTf afforded a mixture of α and β cyclized (1→3)-linked disaccharides. Cleavage of the bridge with NaOMe in MeOH afforded a ratio of β:α of 26:50 3-*O*-D-mannopyranosyl-β-D-glucopyranoside.[57] However, the low β-selectivity and extensive manipulations required render this approach unfavourable in the construction of β-mannopyranosidic linkages.

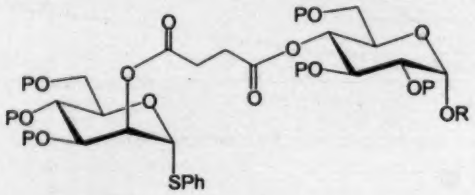


Figure 1.13 Ziegler's extension of IAD using a succinate tether. Taken from T.L. Lowary. [27]

Although excellent success was achieved by Hindsgaul, Stork, and Ogawa, in specific cases, the required mixed acetals and silyl ethers are generally not easily synthesized and the method has not gained wide spread use since the yields are also not especially attractive.

1.3c Recent Advances

Extension of Kahne's sulfoxide glycosylation system by Crich has led to the development of a very successful direct method in the synthesis of β-D-

mannopyranosides with a variety of acceptors (Figure 1.14).[48] Activation of anomeric sulfoxides with Tf_2O in CH_2Cl_2 at -78 °C and subsequent dropwise addition of the desired alcohol in CH_2Cl_2 was highly selective for the β-anomer. The stereocontrol was observed to be dependant on the order of reagent addition.

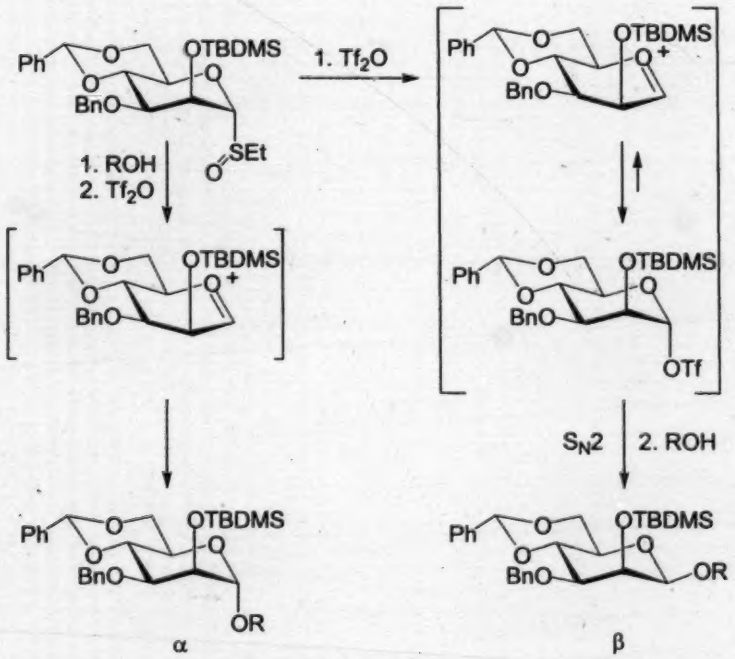


Figure 1.14 Crich's method in forming 1,2-*cis*-β glycosides. Taken from T.L. Lowary.[27]

The donor must first be activated with Tf_2O to expel a sulfinate ester and form the oxocarbenium intermediate.[48] The cation is then trapped by the triflate anion in the absence of other nucleophiles to form the α-triflate, which is then displaced in S_N2 fashion by the incoming alcohol to give the

β-mannopyranoside. DTBMP is a non-nucleophilic base employed to scavenge *in situ* "TfOH". If Tf_2O is added after the alcohol acceptor is mixed with the sulfoxide, the α-anomer is selectively produced as the alcohol nucleophile competes with the triflate anion for the oxocarbenium ion. The use of 4,6-*O*-benzylidene-protected pyranosyl systems further increases the β-selectivity of this method through torsional control. A tightly associated ion-pair between the α-triflate and the oxocarbenium ion is formed as a result of the rigid bicyclic nature of the benzylidene acetal. This is compared to the use of simple 4,6-di-*O*-benzyl congeners which easily form oxocarbenium ions.[48] Crich's approach to β-D-mannopyranosides is currently a leading method.

Direct formation of 1,2-*cis*-β glycosides has also been achieved via a "locked anomeric configuration" by Kovač.[16] The use of 1,2-*cis* -stannylene acetals, pioneered by Schuerch,[60] increases the nucleophilicity of the anomeric oxygen and also locks the anomeric position in the desired stereochemistry. The S_N2 displacement of a secondary triflate is best in polar solvents; however, moderate yields preclude the wide use of this method in the construction of this difficult linkage.

Future developments and improvements of the many glycosylation methods proposed in the construction of 1,2-*cis*-β glycosides may eventually lead to a general, stereoselective, and high yielding protocol.

At present, the method of Crich and the efficiency of oxidation-reduction inversion of configuration of C-2 are the two methods that offer the most practical solution to β-mannopyranoside synthesis. In the last two

decades, attention has been focused on the distinct advantage nature has in creating these and other glycosylation products through the use of stereospecific enzymes.

1.4 Enzymes in Glycoside Metabolism: Classification and their Use in Synthesis

1.4a The Biological Importance of Enzymes

Enzymes are proteins that catalyze chemical reactions vital to life that would occur too slowly or not at all on their own. Their necessity is exemplified by the fact that a single critical malfunctioning enzyme can lead to serious disease.[4] Enzymes provide energetic pathways with reduced activation energy requirements to allow reactions to proceed much more quickly. The stereo-, regio-, and chemoselectivity as well as the specificity of these biocatalysts render them extremely attractive to the synthetic chemist. Protecting group manipulations are avoided and synthesis of complex glycans is achievable.[19] In some instances, a single enzymatic transformation replaces an extensive sequence of chemical reactions.[61] Enzymatic synthesis is a promising and powerful approach for the formation of complex oligosaccharides and glycoconjugates. It is likely that variants of the chemoenzymatic approach, a dualistic strategy, will be responsible for the increased yields, availability, and repertoire of such products in the future. Two classes of enzymes, glycosyltransferases and glycosyl hydrolases, are responsible for the metabolism of glycosidic bonds and the production and alteration of glycoconjugates *in vivo*.[26]

1.4b Glycosyltransferases

Glycosyltransferases catalyze the transfer of a glycosyl acceptor to a donor in oligosaccharide biosynthesis. This donor species is either a sugar nucleotide or a glycosylphospholipid and the transferases that consume these are classified as Leloir and non-Leloir glycosyltransferases respectively.[62] Although few crystal structures of these enzymes are available, two plausible catalytic mechanisms explain the observed retention or inversion of the anomeric configuration.[19] Retention of stereochemistry is explained by a double displacement mechanism that requires the formation of a glycosyl acceptor-enzyme intermediate, the release of the nucleotide in the case of Leloir transferases, followed by nucleophilic attack by the alcoholic acceptor (Figure 1.15).

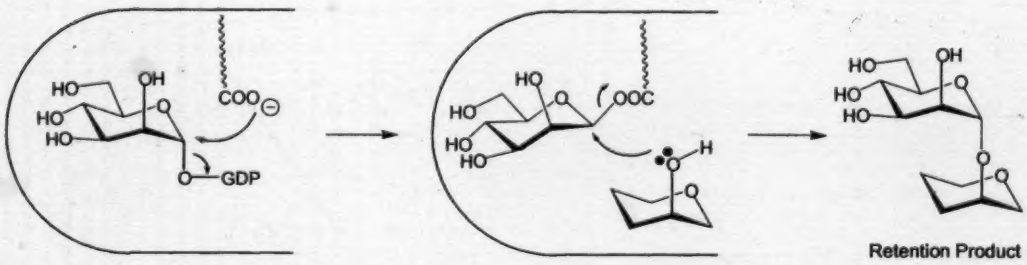


Figure 1.15 Glycosyltransferase retaining mechanism.

Conversely, stereochemical inversion at the anomeric center proceeds more smoothly as a single displacement reaction (Figure 1.16). Deprotonation at the active site promotes the nucleophilic alcohol to attack the C-1 of the glycosyl donor. These mechanisms are speculative but are indirectly

supported by the observation that amino analogs of oligosaccharides often inhibit their respective glycosyltransferases.[63]

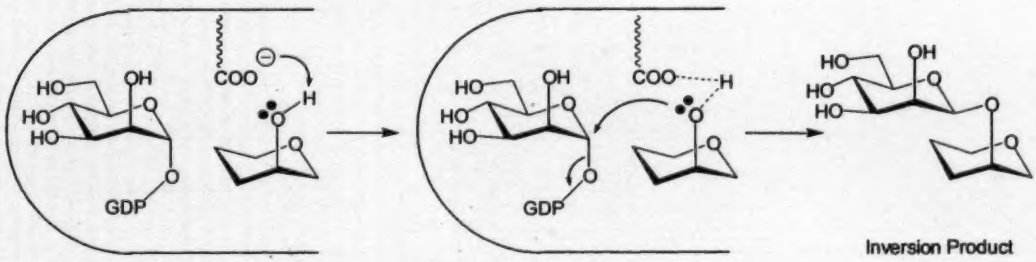


Figure 1.16 Glycosyltransferase inverting mechanism.

Although, glycosyltransferases are highly specific enzymes they often tolerate minor modification in either the donor or acceptor provided that they do not interfere with polar functionalities required for enzyme interaction.[64] Despite the advantages of enzyme use in synthesis, glycosyltransferases are often difficult to purify, handle, and store.[27,61,65] Access to a range of transferases is still restricted; however, a few are commercially available and as time passes recombinant enzymes are being made available and this should eventually address the current scarcity of transferase enzymes. The expense of sugar nucleotides and reaction inhibition by the released nucleoside diphosphates must also be considered. However, the use of *in situ* multi-enzyme sugar nucleotide generation and regeneration systems may alleviate the need for stoichiometric amounts.[66,67] Development of cost efficient methods are still being actively pursued.[68]

1.4c Glycosyl Hydrolases

Glycosyl hydrolases otherwise known as glycosidases naturally hydrolyze anomeric linkages to degrade oligosaccharides.[26] They are classified according to their preference for cleaving internal or terminal glycosidic bonds as *endo*- or *exo*-glycosidases respectively. Glycosidases are readily available and as such many solved crystal structures have revealed that their mechanism of action is analogous to that described for glycosyltransferases. They are classified as retaining or inverting enzymes (Figure1.17a and b).[69] The active site requires the presence of two aspartic acid or glutamic acid amino acid residues for catalysis.[70] The cleaved aglycon is subsequently transferred to a water acceptor.

(a)

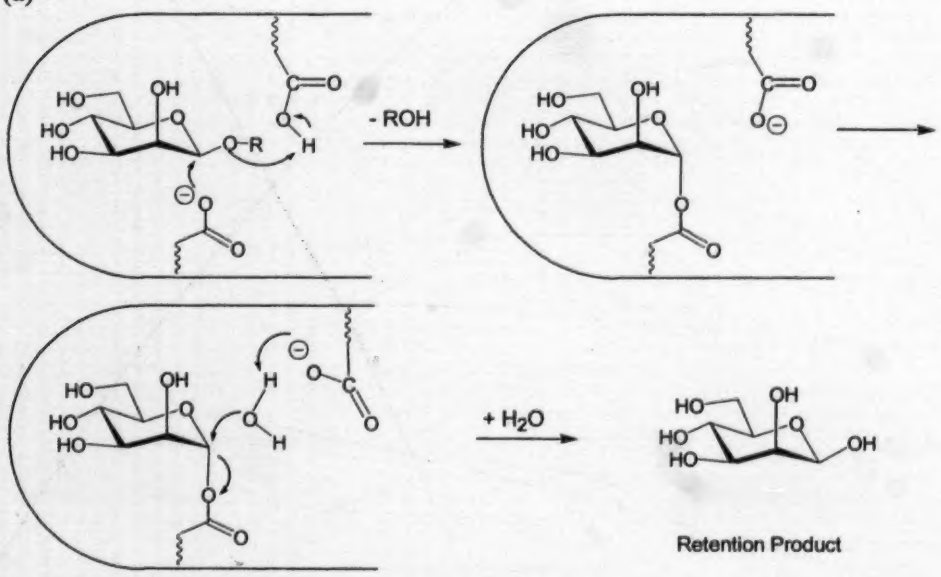


Figure 1.17 (a) Retaining glycosidase mechanism.

(b)

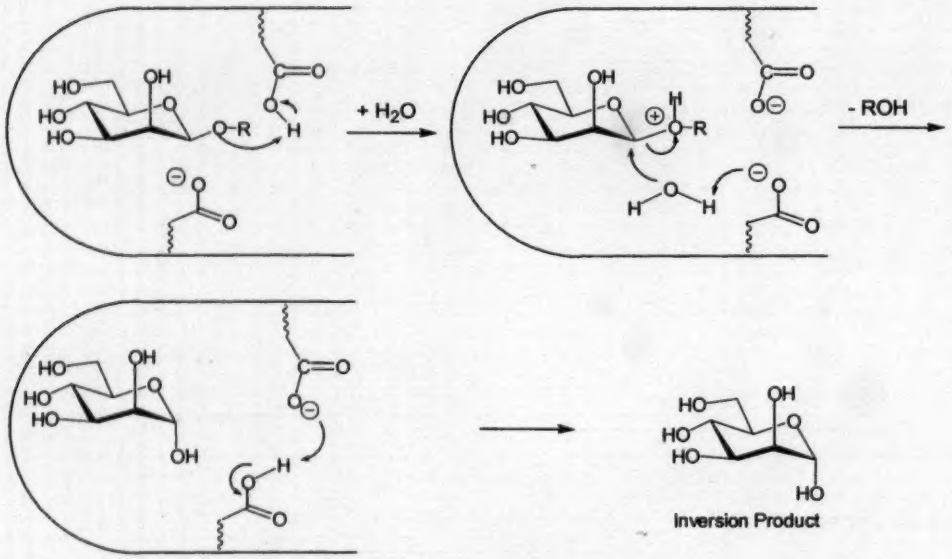


Figure 1.16 (b) Inverting glycosidase mechanism.

Fortuitously, the hydrolytic activity of glycosidases can be reversed to prepare glycosidic bonds instead of degrading them. However, once formed, the saccharide produced becomes a substrate for hydrolysis. The ubiquitous, economical, and robust nature of glycosidases along with their anomeric stereoselectivity makes these enzymes attractive.[26] Their regioselectivity, however, is sometimes promiscuous and hence partially accounts for the low chemical yields observed. Oligosaccharide synthesis with glycosidases can be achieved under thermodynamic or kinetic conditions.[19]

Thermodynamic conditions shift the equilibrium to oppose what is generally regarded as an irreversible exothermic process. Relative success has been achieved by increasing the concentrations of the alcohol acceptor and

sugar donor, minimizing the presence of water with anhydrous conditions, using organic solvents to reduce the activity of water, and increasing the reaction temperature.[71] The simplicity of this approach is impaired by the typically low yields (10-40%) achieved despite development of more efficient protocols.[72]

Kinetically controlled glycosylation reactions using glycosidases show an improvement in yield (40-50%) and are termed transglycosylation reactions.[19] Activated glycosyl donors, such as fluoride and *p*-nitrophenyl glycosides, must be used to efficiently form the "glycosyl-enzyme" intermediate before the preferential transfer to an alcoholic acceptor in place of water. An excess of donor and acceptor is combined with the glycosidase in a low ratio of water to organic solvent.[26] Regioselectivity can be problematic under theses conditions; however, the use of more selective glycosidases or a multi-enzyme approach to degrade unwanted side products can be employed to counteract this problem.[19]

1.4d "Glycosynthases"

The use of mutant glycosidases as glycosyltransferases was recently implemented by Withers and coworkers. It was determined that the replacement of one of the glutamic or aspartic acid residues with non-nucleophilic alanine or serine would render the still correctly folded enzymes hydrolytically inactive.[73,74] Withers, however, realized that these mutant retaining glycosynthases could still produce oligosaccharides in high yields

when presented with an activated sugar donor that mimicked the covalent glycosyl-enzyme intermediate (64-92%) (Figure 1.18).[73]

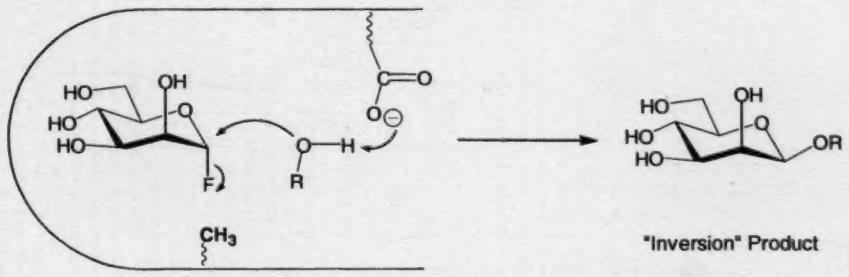


Figure 1.18 "Inverting" mechanism of glycosynthases. Taken from Withers *et al.* [73]

Glycosyl fluorides, activated sugar donors, are quite stable, easily soluble, and readily react with charged nucleophiles in aqueous buffers with minimal decomposition.[75] Recently, a family 2 mannosidase (Man2a) has been transformed into a glycosynthase which provides an effective tool for the synthesis of mannopyranosidic linkages.[76] With the large number of known glycosidases,[77] it may only be a matter of time before a wide range of glycosynthases are designed and a variety of glycosidic linkages are obtained and hence dramatically furthering the discipline of glyobiology. It remains to be seen whether such enzymes can be integrated into the strategy for synthesis of large glycans.

1.5 Scope of the Project

A synthetic conjugate vaccine[78] against the immunologically important (1→2)-*β*-mannan oligomer[79] of the pathogenic yeast *Candida albicans* has been developed. However, a more efficient protocol involving a chemoenzymatic synthesis of the mannan chain could be envisaged if appropriate glycosyltransferases could be identified and expressed as recombinant enzymes. In this thesis, preliminary steps toward the identification of these enzymes in *Candida albicans* are reported. Various mono, di-, and tri- fluorescent glycosyl acceptors containing the challenging 1,2-*cis*-β linkage were prepared using novel and known methodologies. These were subsequently used to probe fungal extracts for β-(1→2)-mannopyranosyltransferase activity. Reaction products were analyzed by capillary electrophoresis,[67] matrix-assisted laser desorption/ionization (MALDI), nuclear magnetic resonance (NMR) and with α- and β-mannosidase assays.

Chapter 2

Immunology of the Mannan of *Candida albicans*

2.1 Introduction to *Candida albicans*

Fungi are gaining importance as opportunistic human pathogens and have recently become the seventh most common cause of infectious disease related deaths in the United States.[80] *Candida* is the fourth most common causative agent of nosocomial bloodstream infections, mainly due to its *albicans* sub-species.[81] *Candida albicans* is ubiquitously found on the mucosal surfaces of the mouth, digestive tract, and genitourinary system of healthy individuals (Figure 2.1). This opportunistic fungal pathogen is the most common etiological agent responsible for causing mild superficial vaginal infections in women and invasive life threatening candidemia in immunocompromised patients. Individuals scheduled to receive abdominal surgery; transplantations, including bone marrow, kidney or heart, immunosuppressive cancer therapy, as well as AIDS patients and those exposed to long term hospitalization are at risk.[82-85] Because of the similarity between fungal and animal cells, many potential antifungal therapeutic targets are inaccessible due to significant cross-reaction toxicity.[86] As such, the number of new antifungal agents available for *in vivo* application is dramatically reduced. Furthermore, an increase in drug resistance combined with the high mortality rates associated with *Candida*, 38% in the United

States and 19-23% in Canada,[87,88] have led to a search for new treatment strategies including conjugate vaccines.

Figure 2.1 The opportunistic fungal pathogen *Candida albicans*. Taken from S.M. Guitiérrez.[89]

2.2 Antibody Production and Conjugate Vaccines

Immunoglobulin M (IgM) receptors, found on the surface of antibody producing cells (B-cell), bind foreign antigen proteins that are encountered in the human body (Figure 2.2).[90] The antigen is internalized by antigen-mediated endocytosis and the proteins are degraded in lysosomes into peptides that are bound by class II major histocompatibility molecules (MHC) and are exported to the B-cell surface where the complex is presented to T-cells. Only helper thymus cells (T-cell) that have been previously sensitized to the same antigen by macrophage or dendritic cells recognize the peptide:MHC class II complex. T-cell sensitivity and subsequent signalling is increased with the binding of the co-receptor CD4 to the peptide:MHC class II complex. The armed T-cell contains two types of effector molecules that synergistically

activate the B-cell. A particularly important membrane-bound effector, the CD40 ligand (CD40L) binds to the B-cell tumor necrosis factor receptor CD40.[91] This interaction is crucial in promoting the resting B-cell into the cell cycle and is vital for B-cell response to thymus-dependant antigens. Secreted effector cytokines, such as interleukin 4 (IL-4), stimulate B-cell proliferation and differentiation into antibody producing plasma cells and memory cells.[90]

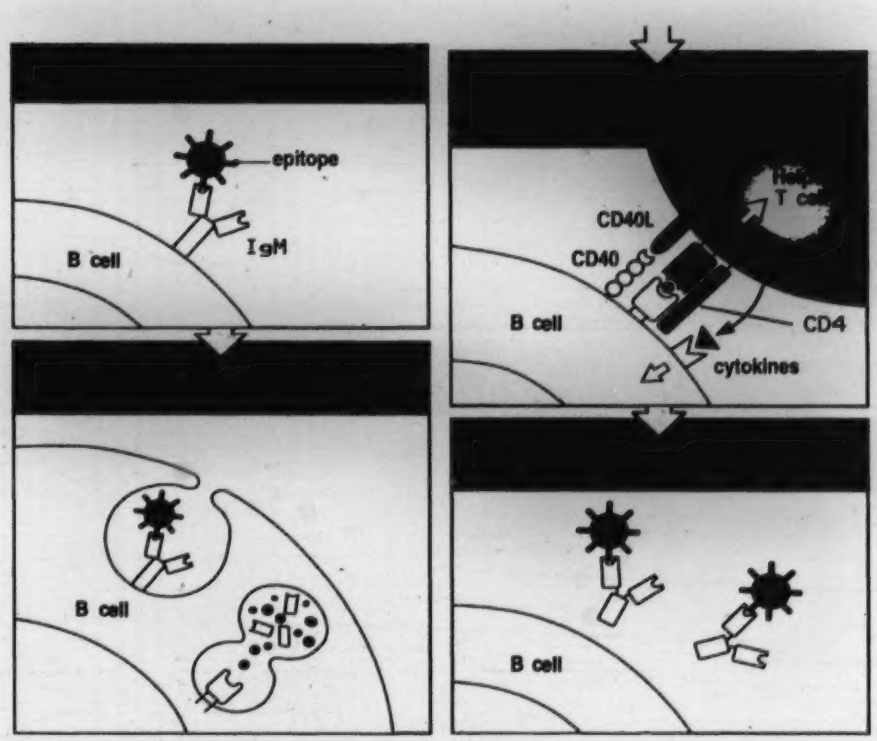


Figure 2.2 B-cell activation by armed helper T-cells. Taken from C. A. Janeway and P. Travers, "Immunobiology: The Immune System in Health and Disease.[90]

Secreted effector cytokines are also responsible for inducing a class switch in immunoglobulin expression from IgM to IgG. [90,92] The production of IgG antibodies is significant as they have a higher affinity for the inducing

antigen and activate different effector mechanisms, thereby increasing the immune response towards the pathogen. Affinity maturation of IgGs is rapidly achieved through somatic hypermutation of the immunoglobulin variable-domain genes of B-cells.[90]

In general, carbohydrates cannot generate an effective immune response because they lack the capacity to recruit helper T-cells. [90] However, coupling of carbohydrates to an antigenic protein, such as bovine serum albumin (BSA) or tetanus toxoid (TT), render these haptens immunogenic as T-cells can be primed against peptides derived from these conjugate proteins (Figure 2.3). B-cells are then activated to produce antibodies against the carbohydrate antigen.

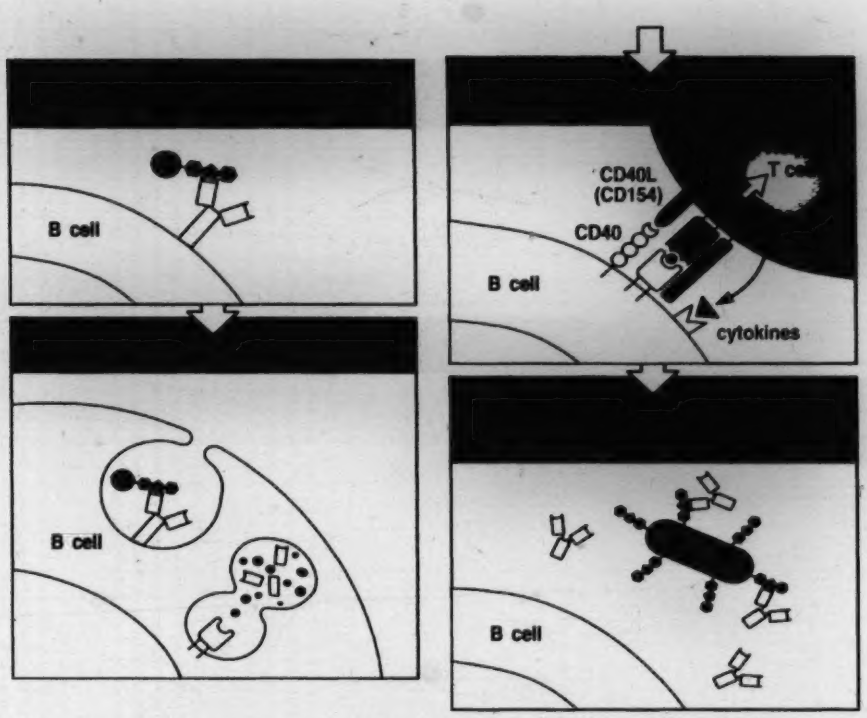


Figure 2.3 Protein antigens attached to polysaccharide antigens allow T-cells to activate polysaccharide-specific B-cells. Taken from C. A. Janeway and P. Travers, "Immunobiology: The Immune System in Health and Disease.[90]

This "linked" recognition strategy in vaccines has proven effective in eliciting a protective response and has been applied in many commercially available vaccines[93] against diseases such as *Haemphilus influenzae* B bacteria responsible for causing meningitis.[94] The same approach is being implemented in the development of a conjugate carbohydrate vaccine against *Candida albicans*.

2.3 The cell wall of *Candida albicans*

As the most external part of the cell, the fungal cell wall is responsible for the initial interaction with its host as well as antigenic expression, adhesion, and cell-cell interactions.[95] This wall-structure is also essential in shielding the cell from osmotic, chemical, and biological harm and, due to its rigidity, maintains the integrity and shape of the cell.

The cell wall of *Candida albicans* is mainly composed of chitin, β-glucan, and phosphomanno-glycoprotein (Figure 2.4a). Content ratio between these three components is dependant upon the current growth form the fungus has adopted.[96] Microfibrillar polymers of chitin and β-glucan form a rigid mesh that impart physical strength to the cellular skeleton (Figure 2.4b).[97] Phosphomanno-glycoproteins have received the most attention, however, as they are highly immunogenic and are able to play a role in cell mediated immunity and humoral immune response.[97]

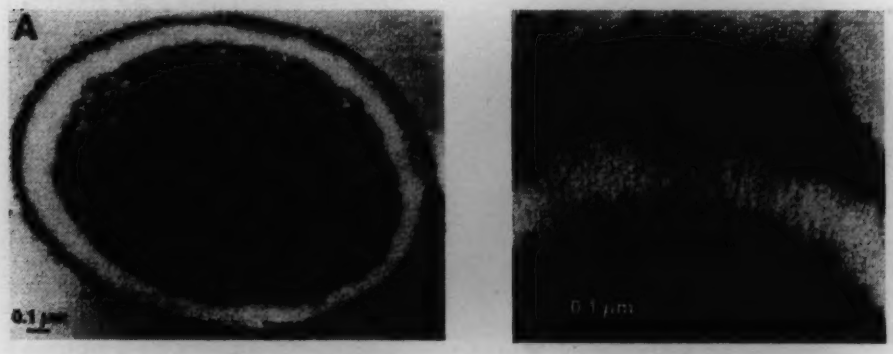


Figure 2.4 Transmission electron micrographs of ultrathin sections of *Candida albicans* fixed with DMSO. (A) A whole yeast cell (B) Section of a yeast cell showing details of the cell wall. Taken from M.A. Jabra-Riz *et al.*[98]

Suzuki has determined that *C. albicans* serotypes A and B are defined by surface mannan composition.[99] Structural elucidation of the cell wall phosphomanno-glycoprotein has been accomplished (Figure 2.5).[100-102] The glycan portion of the complex *N*-linked glycoprotein is composed of an extended α-(1→6)-D-mannopyranan backbone containing α-(1→2)-D-mannopyranan oligomer branches.[101] Residues of α-(1→3)- and α-(1→6)-linked mannan can also be found within these side chains.[103] Short β-(1→2)-D-mannopyranan oligomers (n = 1-2) covalently cap the non-reducing terminal sites of some of the α-(1→2)-D-mannopyranan side chains to form antigenic factor 6.[101,104] Factor 6 includes both the α- and β-mannose residues. In addition to the acid stable structure, a β-(1→2)- D-mannopyranan oligomer is linked to an α side chain through an acid labile phosphodiester bond to form antigenic factor 5. The exact point of attachment has yet to be determined.[100] Serotype B is characterized by the presence of only antigenic factor 5 whereas both factors are present in serotype A.

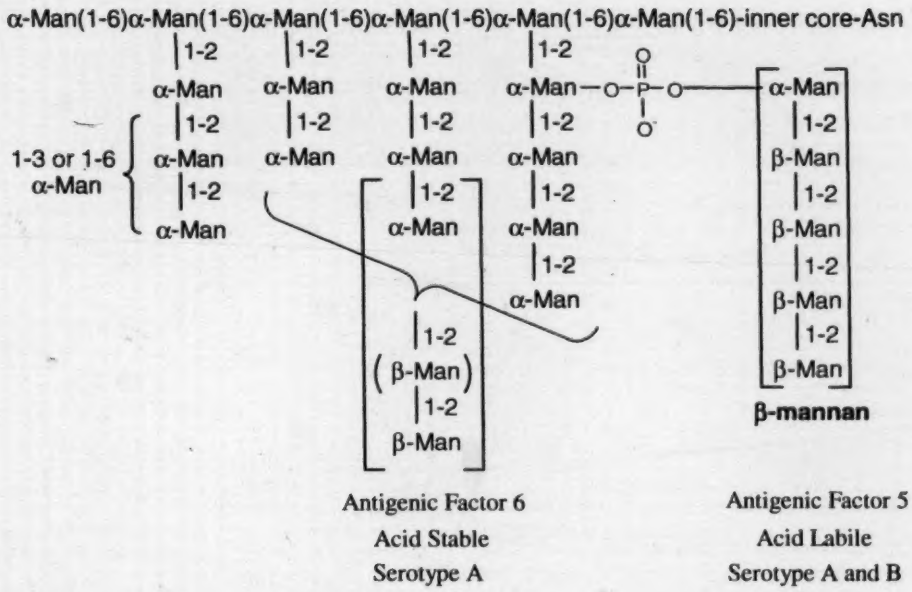


Figure 2.5 Structure of the cell wall phosphomannan antigens.[100-102,104]

2.4 Current Immunological Data

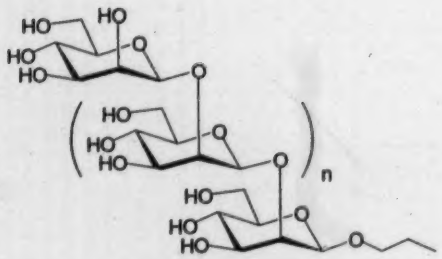
Conflicting reports regarding the role of antibodies against candidiasis have emerged. The presence of antibodies against human and *Candida* heat shock protein (HSP) 90 in patients with acquired immune deficiency syndrome (AIDS) has correlated with recovery from infection and further protection against oropharyngeal and disseminated candidiasis.[105,106] Surprisingly, antibody titers against *Candida albicans* have been found in greater levels in patients with candidiasis compared to controls and these titers were also observed to increase in cancer patients dying of candidiasis.[107] The occurrence of protective and non-protective antibodies has become apparent from Cutler's results.

Compelling evidence indicated that monoclonal antibodies of the IgM isotype against antigenic factor 5 (acid labile β-mannose chains) offered protective immunity against vaginal and disseminated candidiasis in mice; however, antibodies raised against antigenic factor 6 (β-mannose attached to α-mannose residues) were not protective.[108] These findings demonstrated that the cell wall displays epitopes that may or may not lead to the production of protective antibodies. Hence, the presence of antibodies against *Candida* fungal cells in patients or in mice does not predict the resistance potential of these individuals. Furthermore, the mannan epitopes are found to be distributed unevenly across the cell surface.[109] The mannotriose in antigenic factor 5 was spread evenly over the yeast cell surface; whereas, antigenic factor 6 had a much more patchy distribution. An interpretation of these results suggests that the acid stable epitope in antigenic factor 6 is proximal to the cell surface and may be partially blocked by acid labile phosphomannan components. As a possible result, patients may not develop antibodies of the correct specificity or in low titers due to the extremely complicated antigenic nature of the cell wall of *Candida albicans.*[109]

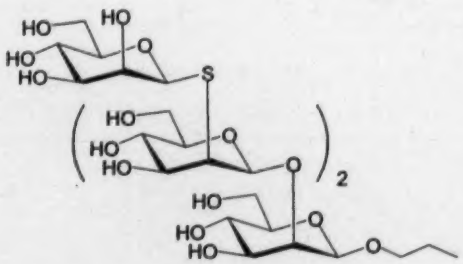
Cutler greatly improved his anti-*Candida albicans* vaccine through the covalent conjugation of acid labile phosphomannan extracts to BSA.[110] Resistance against disseminated candidiasis increased as exemplified by the significant change in challenged mouse survival times from 32-46 days to 65 days. Furthermore, only a priming immunization with a subsequent booster was required as opposed to the previously required four sequential

immunizations with the original liposomal vaccine. IgG and IgM monoclonal antibodies specific for the acid labile mannan were generated from immunized mice and both isotypes are known to confer immunity; however, the individual protective effect is still under investigation. Elucidation of the mannan epitope that is recognized by the protective antibody will improve the design of the conjugate vaccine in the future.[110]

As a result of the immunological data of the extracted β-(1→2)-mannan antigen and its identification as a key epitope in a conjugate vaccine, defined synthetic oligomers were synthesized to probe the immunochemistry of antibodies against antigenic factor 5.[111] A series of di- to hexasaccharide β-(1→2)- mannopyranosides were prepared by Nitz[112] (Figure 2.6) and their chemical synthesis is described in section 2.1f.



(1) Disacharide n = 0
(2) Trisaccharide 1
(3) Tetrasaccharide 2
(4) Pentasaccharide 3
(5) Hexasaccharide 4



(6) Thio-linked Tetrasaccharide

Figure 2.6 A series of di- to hexasaccharide (1→2)-β-mannopyranosides prepared by Nitz. Taken from M. Nitz et al.[112]

These oligomers were evaluated for the inhibition of IgM B6.1 and IgG C3.1 protective antibodies using competitive Enzyme-Linked Immunosorbent Assay (ELISA) (Table 2.1).[112] Surprisingly, the IgM showed highest affinity for disaccharide **1** and trisaccharide **2** and decreasing inhibitory power in the order of tetrasaccharide **3** > pentasaccharide **4** > hexasaccharide **5** (Figure 2.7a). The thio-linked tetrasaccharide **6** was found to have an inhibitory power in between that of the tri- and tetrasaccharide. The same panel of oligomannosides **1-6** tested with IgM B6.1 antibodies revealed similar affinity trends with IgG C3.1. Compounds **1** and **2** again had the highest antibody affinities but the affinity was increased 5- and 2-fold respectively (Figure 2.7b).[112]

Table 2.1 Inhibition of the binding of monoclonal antibodies B6.1 (IgM) and C3.1 (IgG) to *C. albicans* mannan antigen by synthetic oligosaccharides **1–6**. Taken from M. Nitz.[112]

Inhibitor	mAb B6.1		mAb C3.1	
	IC_{50} μM	Relative Potency	IC_{50} μM	Relative Potency
Disaccharide **1**	44	86	8	100
Trisaccharide **2**	38	100	16	50
Tetrasaccharide **3**	108	35	84	10
Pentasaccharide **4**	770	5	421	2
Hexasaccharide **5**	>1000	< 4	844	1
Thio-linked tetrasaccharide **6**	64	60	74	11

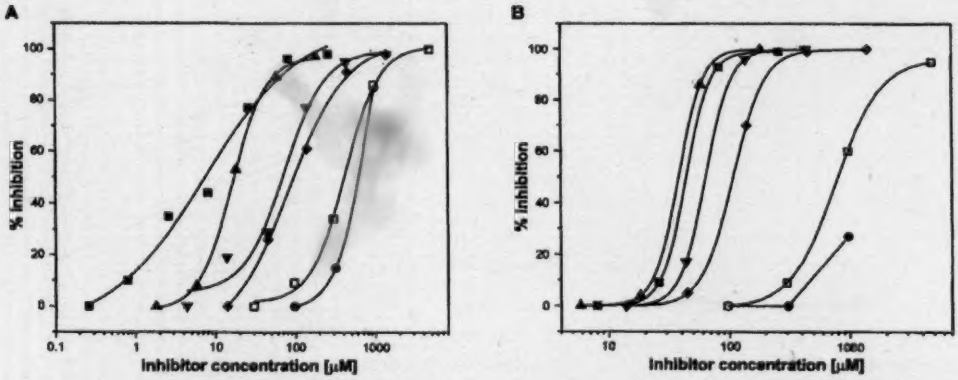


Figure 2.7 ELISA inhibition data for monoclonal antibodies IgM B6.1 (a) and IgG C3.1 (b). M. Nitz *et al.*[112]

Inhibition by synthetic oligosaccharides **1**-**6** of monoclonal antibody binding to *C. albicans* extract. ■, propyl β-(1→2)-D-mannopyranobioside (**1**); ▲, propyl β-(1→2)-D-mannopyranotrioside (**2**); ▼, propyl (1-thio-β-D-mannopyranosyl)-(1→2)-β-D-mannopyranotrioside (**6**); ♦, β-(1→2)-D-mannopyranotetroside (**3**); □, β-propyl (1→2)-D-mannopyranopentoside (**4**); ●, propyl β-(1→2)- D-mannopyranohexoside (**5**).

Solution NMR and molecular modeling studies were consistent with a helical conformation for β-(1→2)-linked pyranomannans.[112] Hence, antibodies that only recognize two to three terminal oligomers may experience steric clashes when binding longer oligomers due to the proximity of the fourth sugar to the first residue or the protein surface. Therefore, saccharides may be forced to adopt higher energy conformations to relieve this conflict. The third residue repeat of this helix coincided with the optimal inhibitory activity of a trisaccharide epitope. This hypothesis is consistent with the observed increased antibody affinity of **6**, with its thio-linked terminal residue, over *O*-linked analog **3**. This is an atypical observation as thio-glycoside mimetics usually bind their protein receptors with lower affinity.[113] The

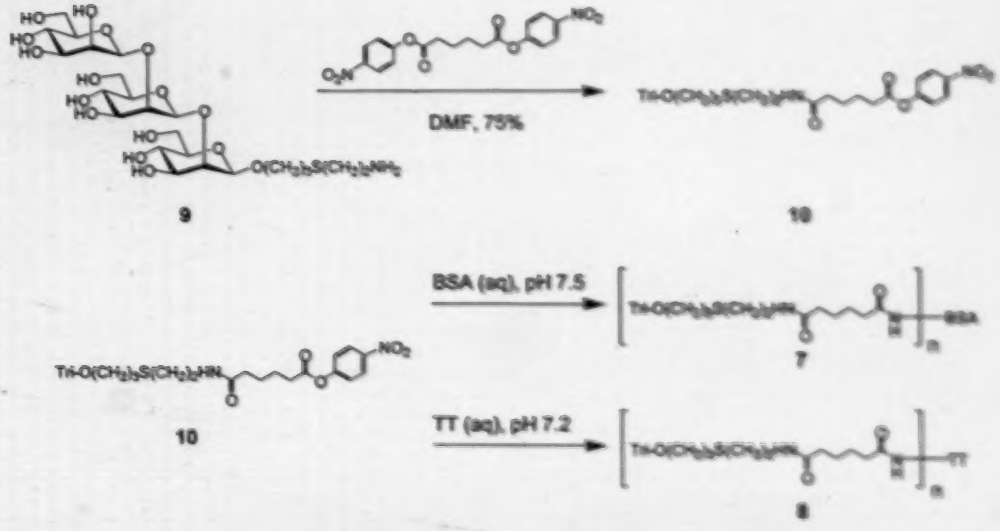
flexibility of the thioglycosidic bond allows the terminal residue to relieve some of the steric interactions with walls of the antibody-binding site by adopting an alternative energy conformation. Although Shiabata and co-workers have proposed that the size of the recognition site is limited to four mannose residues, β-Man-(1→2)-β-Man(1→2)-α-Man-(1→2)-Man,[102] Nitz has demonstrated that protective IgG and IgM monoclonal antibodies that recognize antigenic factor 5 are best inhibited by di- and trisaccharide β-(1→2)- mannopyranosides.[112]

2.5 Conjugation Strategies and Associated Immunological Data

The conjugation strategy implemented to covalently link an oligosaccharide to a protein to produce a conjugate vaccine is a determining factor in the synthetic approach to oligosaccharide assembly and deprotection. Furthermore, the chemical nature of the tether may impart undesirable immunological properties to the vaccine. For example, the use of the efficient homo-bifunctional reagent, diethyl squarate, in conjugate vaccines has been associated with a reduced immune response to the oligosaccharide epitope[114] and with a potential immune response to the squarate residue.[115]

Recently, an easily synthesized and novel aliphatic chain linker[116] was employed in the production of a β-(1→2)-trimannoside glycoconjugate vaccine **7** and **8** (Scheme 2.1).[117] The *p*-nitrophenyl ester of the homo-bifunctional adipic acid coupling reagent[118] was reacted efficiently (75%) with an amino terminated tether of an oligosaccharide **9** under mild conditions

giving **10** (Scheme 2.1a). The remaining activated ester of the linker was then coupled to BSA or TT by an 18 hour incubation in phosphate buffered saline (PBS) at ambient temperature (Scheme 2.1b). Through MALDI-TOF mass spectroscopy, it was determined that 12 ligands were incorporated by each molecule of BSA or TT resulting in 44% and 33% conjugation efficiency respectively.[117]



Scheme 2.1 Novel aliphatic chain linker in the preparation of a β-(1→2)-trimannoside glycoconjugate vaccine. Taken from X. Wu and D. Bundle.[117]

The immune response in rabbits to immunization with trisaccharide antigen **8** conjugated to TT has recently resulted in titers of 1:272,000 against the synthetic antigen and 1:64,000 against the native *Candida albicans* cell wall mannan antigen (Figure 2.8).[119]

Figure 2.8 Immunofluorescent staining of *C. albicans* cells using rabbit antiserum raised against the trisaccharide conjugate. Antibodies bind to the antigen presented on the walls of *Candida* hyphae and budding cells. (D. Bundle and R. Rennie, unpublished results).[120]

2.6 Summary

Certain criteria for a synthetic conjugate vaccine against *Candida albicans* have been established. A synthetic trisaccharide epitope coupled to a carrier protein that can be used in humans and is capable of inducing a strong carrier-enhanced antibody response is required. Furthermore, covalent attachment of the saccharide to a protein necessitates the use of a non-immunogenic linker so that hapten response is confined to the sugar epitope. If this is achieved, we can maximize efficacy of the Candida vaccine. These requirements will dictate the chemistry employed in the tether and in the structural conformation of the oligosaccharide.

CHAPTER 3

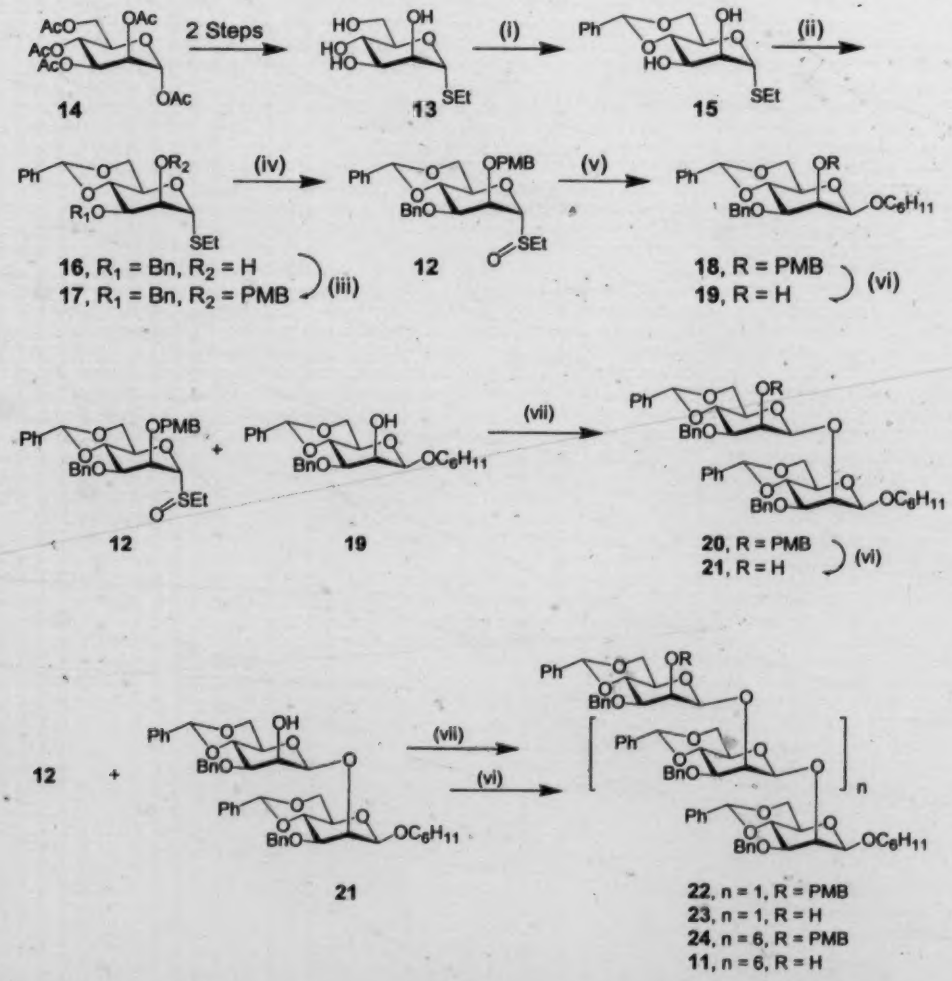
Chemical Synthesis of Mannopyranosides found in *Candida albicans*

3.1 Previous Synthesis of β-(1→2)-Mannopyranosides

A long standing challenge in organic chemistry has been the stereoselective synthesis of β-(1→2)-mannopyranosides for reasons explained in section 1.2d. Although many advances in the production of 1,2-*cis*-β linkages have been made, a general method has not been found.[41,42,46,53] Four syntheses of β-(1→2)-mannopyranosides have been achieved by Crich and coworkers,[48,121] Nitz and Bundle,[111] Wu and Bundle,[117] and Fraser-Reid and coworkers[10].

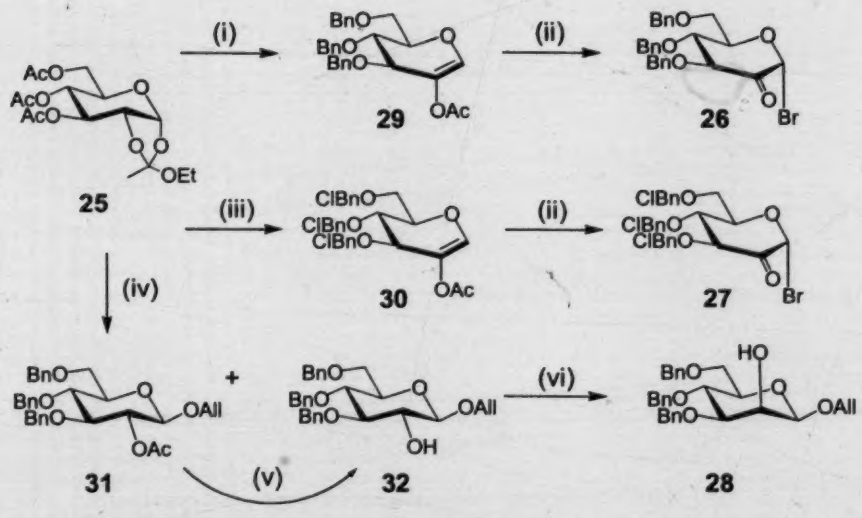
Crich's strategy extends from Kahne's[22] sulfoxide glycosylation approach and is the only direct method employed in β-(1→2)-mannopyranoside synthesis. The general strategy is described in detail in section 1.3c. Specifically, as seen in Scheme 3.1, the synthesis of ocatmer **11** hinged upon the formation of a selectively protected and diastereomerically pure sulfoxide donor **12**.[121] The ethyl α-thiomannoside **13** was prepared in two steps from peracetylated α-D-mannopyranose **14** and converted to the 4,6-*O*-benzylidene derivative **15**. Further selective benzylation of O-3 in **16** and para-methoxybenzylation (PMB) of O-2 in **17** and subsequent oxidation with oxone (90%) yielded the desired donor **12**. Introduction of a cyclohexanol aglycon moiety **18** and selective removal of the PMB group with DDQ

provided acceptor **19**. Compound **12** was coupled to **19** with Tf_2O in the presence of TTBP at -78 °C to give **20** in 94% yield. Selective PMB removal afforded acceptor **21** and glycosylation gave trisaccharide **22** (89%). Deacetylation of **23** and further reiteration of this cycle eventually gave octamer **11** in 64% yield with only 4.5:1 β/α selectivity.[121]



Scheme 3.1 Crich's β-(1→2)-mannopyranoside synthesis. (i) benzaldehyde dimethyl acetal, p-TSA (ii) a) dibutyltin oxide b) benzyl bromide (iii) p-methoxybenzyl chloride, NaH (iv) oxone (v) Cyclohexanol, Tf_2O, TTBP (vi) DDQ (vii) Tf_2O, TTBP. Taken form Crich *et al.*[121] and references within.

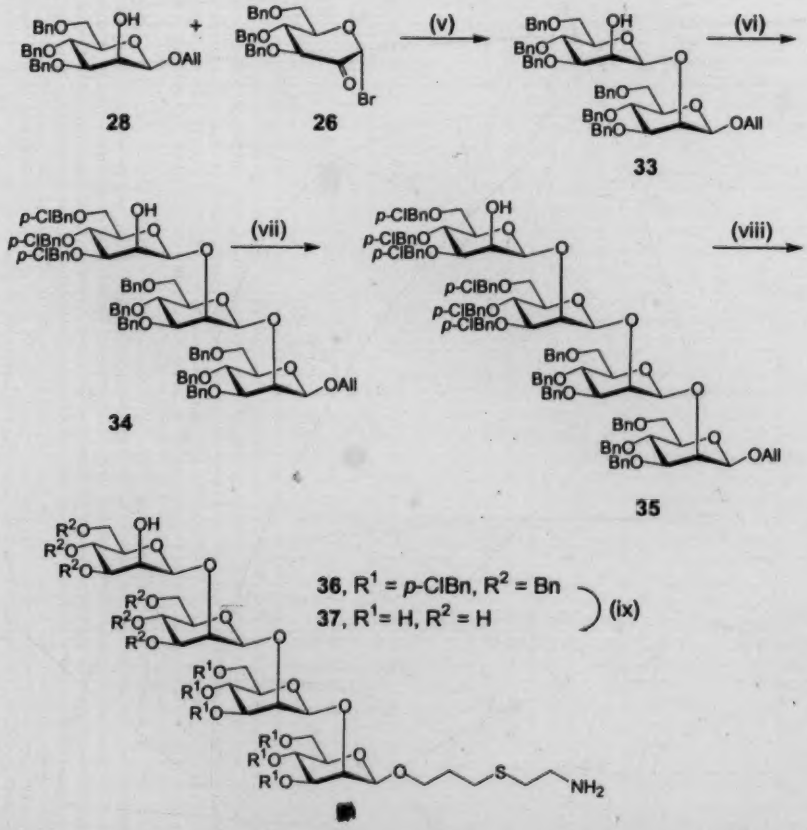
Nitz was the first to develop an indirect approach for the synthesis of β-(1→2)-mannopyranosides involving the use of Lichtenthaler's ulosyl bromide methodology[46] (section 1.3a).[111] As shown in Scheme 3.2, the acetylated *O*-ethyl orthoester **25** is a common precursor to the formation of both the ulosyl bromide donors **26** and **27** as well as the benzylated allyl mannosyl acceptor **28**. Compound **25** is easily obtained in two steps from the peracetylated α-D-mannopyranose.[122] Compound **25** was subject to benzylation or *p*-chlorobenzylation and subsequent thermal rearrangement in bromobenzene afforded **29** and **30** respectively. Further treatment with NBS and EtOH in CH_2Cl_2 gave the desired ulosyl bromides **26** and **27**. Orthoester **25** was first benzylated and elaborated by Lewis acid promoted glycosylation in allyl alcohol to give an equal mixture of **31** and **32**. Zemplen deacetylation of the mixture followed by Swern oxidation and reduction with $NaBH_4$ gave allyl β-D-mannopyranoside **28** in good yield and high stereoselectivity.[111]



Scheme 3.2 Formation of donors **26**, **27** and acceptor **28** for use in Nitz's β-(1→2)-mannopyranoside synthesis. (i) a) BnBr, KOH b) bromobenzene, pyridine (ii) NBS (iii) a) NaOMe b) p-chlorobenzyl chloride, NaH c) bromobenzene, pyridine (iv) a) BnBr, KOH b) BF_3-OEt_2, allyl alcohol (v) NaOMe (vi) a) Ac_2O, DMSO b) $NaBH_4$. Taken from M. Nitz *et al.*[111]

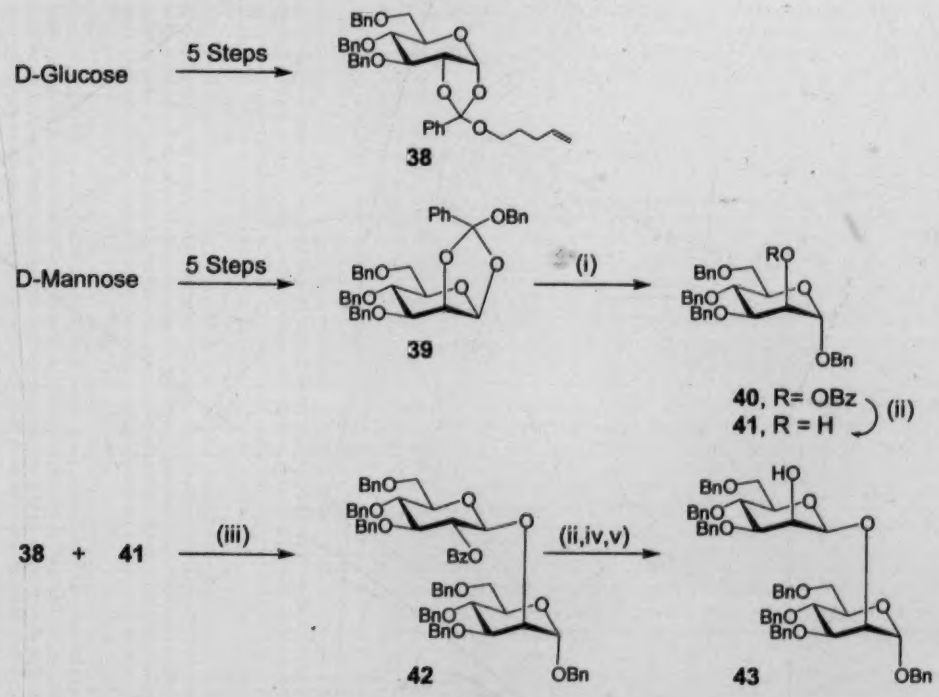
Glycosylation of acceptor **28** with donor **26** in the presence of silver zeolite followed by stereoselective reduction with L-Selectride rendered **33** in 78% yield (Scheme 3.3).[111] Sequential introduction of β-mannopyranosyl units was achieved using the *p*-chlorobenzyl donor **27**. Activation with AgOTf and DTBMP in acetonitrile with further selective reduction with L-Selectride gave glycosyl acceptor trisaccharide **34**. Tetrasaccharide **35** was obtained in 48% yield under the above activation conditions in the sterically more hindered pivaloyl nitrile solvent. This solvent minimized attack by the alcohol on the intermediate nitrile to give a complex imidate. The synthesis of a penta- and hexasaccharide were also described in a latter publication under these conditions but with decreased stereoselectivity (4:1 β/α).[78] The aglyconic allyl

group of **35** provided an attachment point for the photoaddition of 2-aminoethanethiol, a versatile amine terminated functionality via which immunogenic proteins can be attached. Formation of **36** was achieved under radical ultraviolet irradiation conditions (365 nm) in a quartz vessel in 74% yield in the presence of benzyl protecting groups. Deprotection of **36** was accomplished with Birch reduction conditions to give **37** in 77% yield.[111]



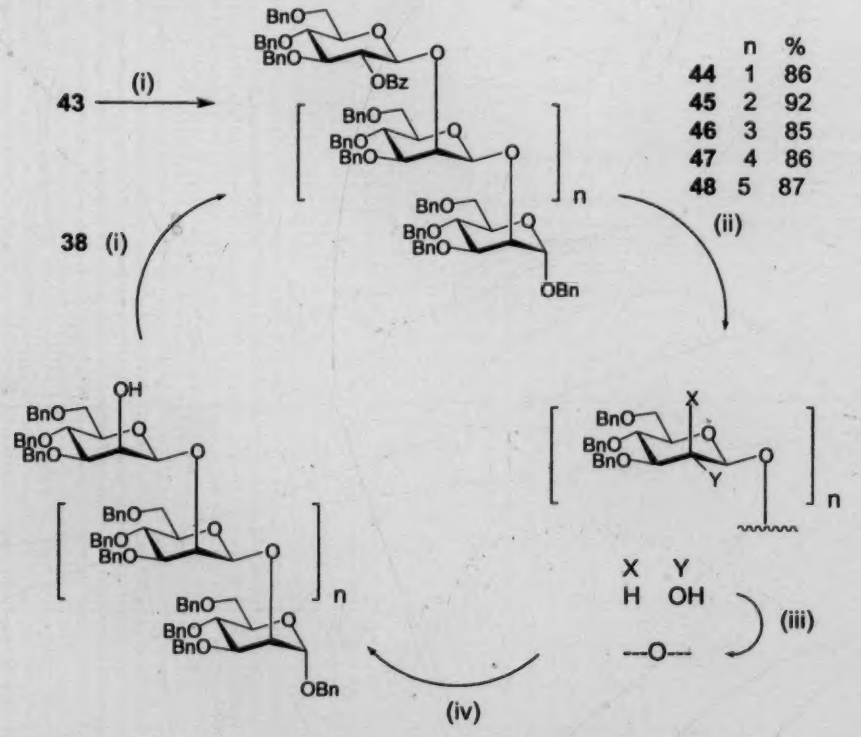
Scheme 3.3 Nitz's β-(1→2)-mannopyranoside synthesis. (v) a) Ag zeolite, CH_2Cl_2 b) L-Selectride (vi) a) **27**, AgOTf, DTBMP, CH_3CN b) L-Selectride (vii) a) **27**, AgOTf, DTBMP, $(CH_3)_3CCN$ b) L-Selectride (viii) 2-aminoethanethiol hydrochloride, *hv* 365 nm (ix) Na, NH_3. Taken from M. Nitz *et al.*[111]

Fraser-Reid designed an indirect iterative synthesis that uses an n-pentenyl orthoester (NPOE) based glycosyl donor approach in conjunction with an oxidation-reduction sequence first proposed by Kotchetkov[123] and Ekborg.[10,124] Two key glucosyl **38** and mannosyl **39** orthoesters were easily obtained in five identical steps using 4-pentenol and benzyl alcohol respectively (Scheme 3.4). Treatment of **39** with acidic TBDMSOTf gave the rearranged benzyl glycoside **40**, which upon debenzoylation gave **41** to constitute the reducing end of the future oligomannosides. Coupling of NPOE donor **38** to **41** mediated by TBDMSOTf and NIS **42** and sequential saponification, oxidation, and reduction forms **43**.[10]



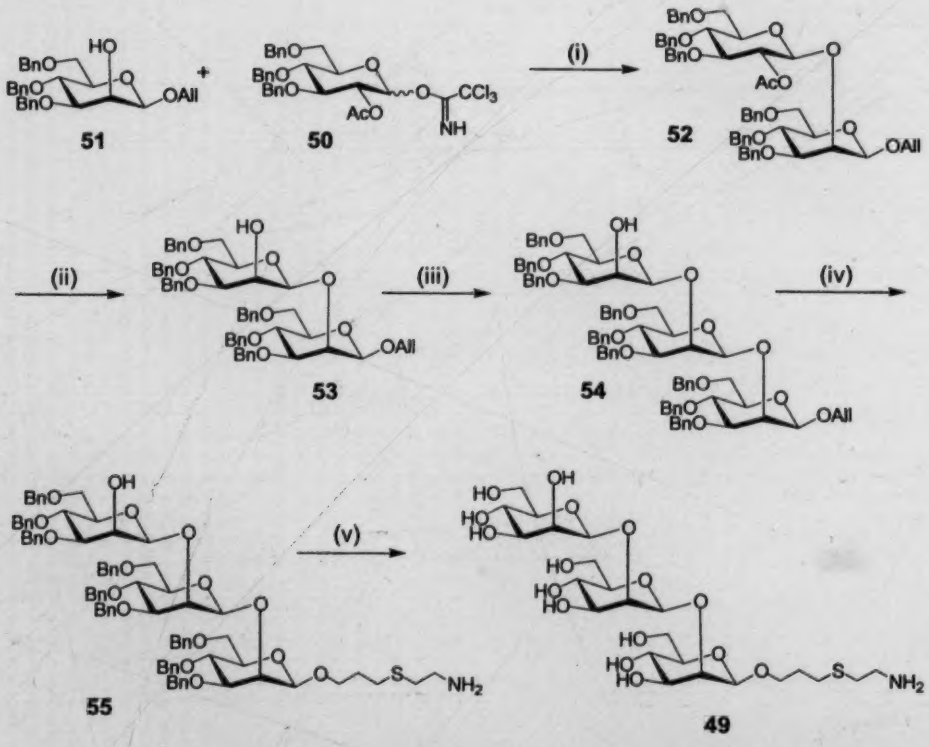
Scheme 3.4 Formation of donor **38** and acceptor **41** and their use in β-(1→2)-mannopyranoside synthesis. (i)TBDMSOTf (ii) NaOMe (iii) NIS, TBDMSOTf (iv) Swern (v) L-Selectride. Taken from Fraser-Reid *et al.*[10]

Acceptor **43** was then introduced into the first iteration of the cycle seen in Scheme 3.5.[10] Selective deprotection, oxidation, and reduction followed by another glycosylation step completed the cycle to give **44**. As the synthesis progressed, however, excess of the easily obtained donor **38** was used to ensure complete glycosylation of the growing elaborated acceptor. Excellent yields and stereoselectivities were also achieved with this glycosylation method as only trace amounts of the *cis*-coupled product were detected by ^{1}H NMR.[10]



Scheme 3.5 The iterative cycle in Fraser-Reid's synthesis of β-(1→2)-mannopyranosides. (i)TBDMSOTf, NIS (ii) NaOMe (iii) Swern (iv) L-Selectride. Taken from Fraser-Reid *et al.*[10]

Wu and Bundle[117] proposed a related but simplified approach to Fraser-Reid's synthesis with a conveniently protected glucopyranosyl trichloroacetimidate donor[125,23] and a subsequent oxidation-reduction sequence.[123,124] Synthesis of trisaccharide **49** was accomplished as outlined in Scheme 3.6. Building block **50** was key to this synthesis and proved to be the compound of divergence in its role of glycosyl donor and further transformation into the glycosyl acceptor **51**. Both **50** and **51** were produced according to published literature in five and nine steps, respectively.[78,125,126] Trichloroacetimidate donor **50** was activated with TMSOTf in CH_2Cl_2 and glycosylated **51** in excellent yield (93%) to give **52**. Deacetylation under Zemplen conditions followed by oxidation with Ac_2O in DMSO and reduction with L-Selectride afforded disaccharide **53** in 88% yield. Repetition of the glycosylation with **50** and reiteration of the saponification, oxidation, and reduction sequence gave **54** in 62% yield over the four steps. Excellent stereoselectivity was achieved as only trace amounts of the β-glucosyl epimer were observed by ^1H NMR. Addition of the terminal amine handle provided **55** and total deprotection, by Birch reduction, yielded trisaccharide **49**.[117]



Scheme 3.6 Previous synthesis of β-1,2-mannopyranosides via a glucosyl donor. (i) TMSOTf (ii) a) NaOMe b) Ac_2O, DMSO c) L-Selectride (iii) a) **39**, TMSOTf b) NaOMe c) Ac_2O, DMSO d) L-Selectride (iv) 2-aminoethanethiol hydrochloride, *hν* 365nm (v) Na, NH_3. Taken from X.Y. Wu and D.R. Bundle.[117]

3.1a Scope of the Project

A cost effective method for the production of a vaccine that avoids multiple synthetic transformations could be based on a chemoenzymatic strategy using native glycosyltransferases. However, no suitable mannopyranosyltransferases have yet been identified. It is therefore necessary to screen *C. albicans* for the appropriate enzymes. To facilitate this approach, we synthesised a series of oligomannosides and elaborated them with a fluorescent moiety (**1-6**) to probe the enzymatic activity of *C. albicans* fungal

extracts in the hope of identifying the desired transferases. Although the synthesis was based on the strategy published by Wu and Bundle, several modifications to the synthetic and coupling strategy were made.

3.2 Synthesis of Acceptors

3.2a Retrosynthetic Analysis

Analysis of the mannan side chain component of the cell wall of *Candida albicans* suggested the construction of six glycosides to identify and isolate the glycosyltransferases that produce the acid stable and acid labile β-mannan epitopes *in vivo* (Figure 3.1). Only the strategy in constructing the β-linkage will be discussed here as the 1,2-*trans*-α-anomer synthetic methodology is well established and is not expected to be challenging.

Attachment of a fluorescent moiety to a tether was essential to the design of the glycoside synthesis as ultra-sensitive fluorophore capillary electrophoresis (CE) based assays would be used in the search for the products of glycosyltransferases. Although a variety of tethers are available for coupling the glycoside to the succinimidyl ester portion of fluorescein-5-ex-succinimidyl ester (Figure 2.9) via a free amine, the allyl group was first identified as a provisionally stable anomeric intermediate that could be functionalized to the amine through photoaddition of 2-aminoethanethiol in the latter stage of synthesis. Second, a 6-azidohexyl aglycon was also proposed due to its heterofunctionality and stability under the manipulation

conditions required in the formation of the β-mannopyranosyl acceptor as well as easy conversion to the free amine under general deprotection conditions.

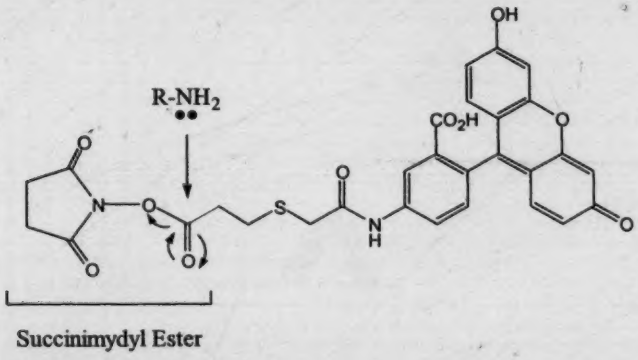


Figure 3.1 Coupling of the free amine to the succinimidyl ester portion of fluorescein-5-ex-succinimidyl ester.

Facile preparation of β-mannopyranosides compared to α-mannopyranosides has been synthetically defiant despite several new approaches presented in section 1.3c. Despite certain advantages offered by direct mannosylation through IAD and Crich's methods, we preferred synthetic routes that involved C-2 epimerization through either oxidation-reduction or ultrasound promoted nucleophilic inversion in the production of the glycosyl acceptor **A** from the common intermediate **B** as most suitable for larger scale synthesis (Figure 3.2). Ultrasonic S_N2 displacement of the gluco-triflate with an acetate nucleophile was attractive due to the elimination of the required photoadditon reaction in the elaboration of the allyl glycoside to the free amine. The synthesis of **B** could be accomplished through a glycosylation strategy from **C** or through an orthoester rearrangement of **D**.

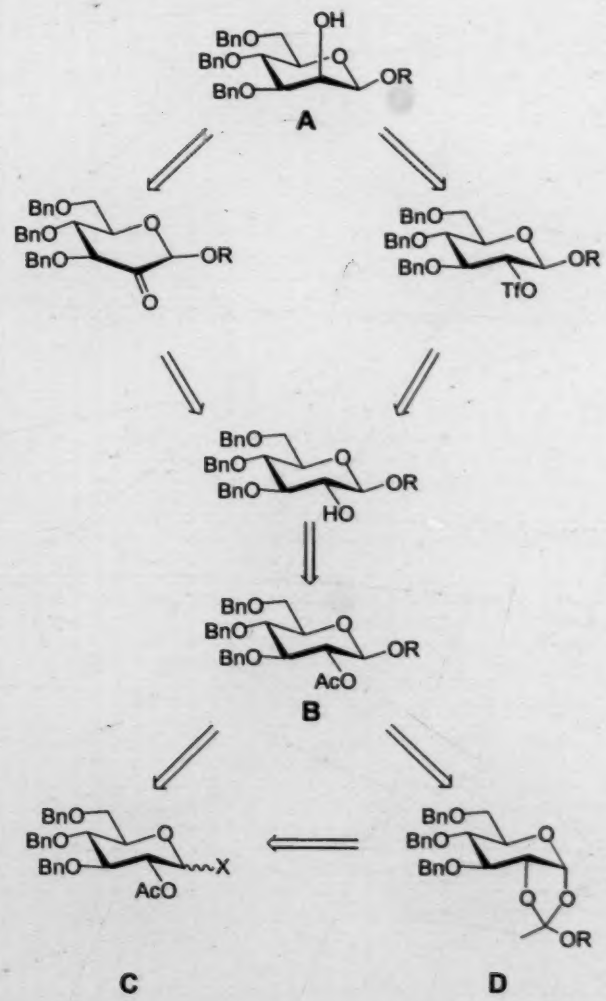


Figure 3.2 Retrosynthetic analysis of the reducing end acceptor.

Extension of the mannosyl residue to form disaccharide **E** can be accomplished by activation of glycosyl donor **C** to acceptor **A** (Figure 3.3). Deprotection to **F** and subsequent C-2 inversion can then be pursued as discussed above to form the desired β-(1→2)-mannopyranoside **G**.

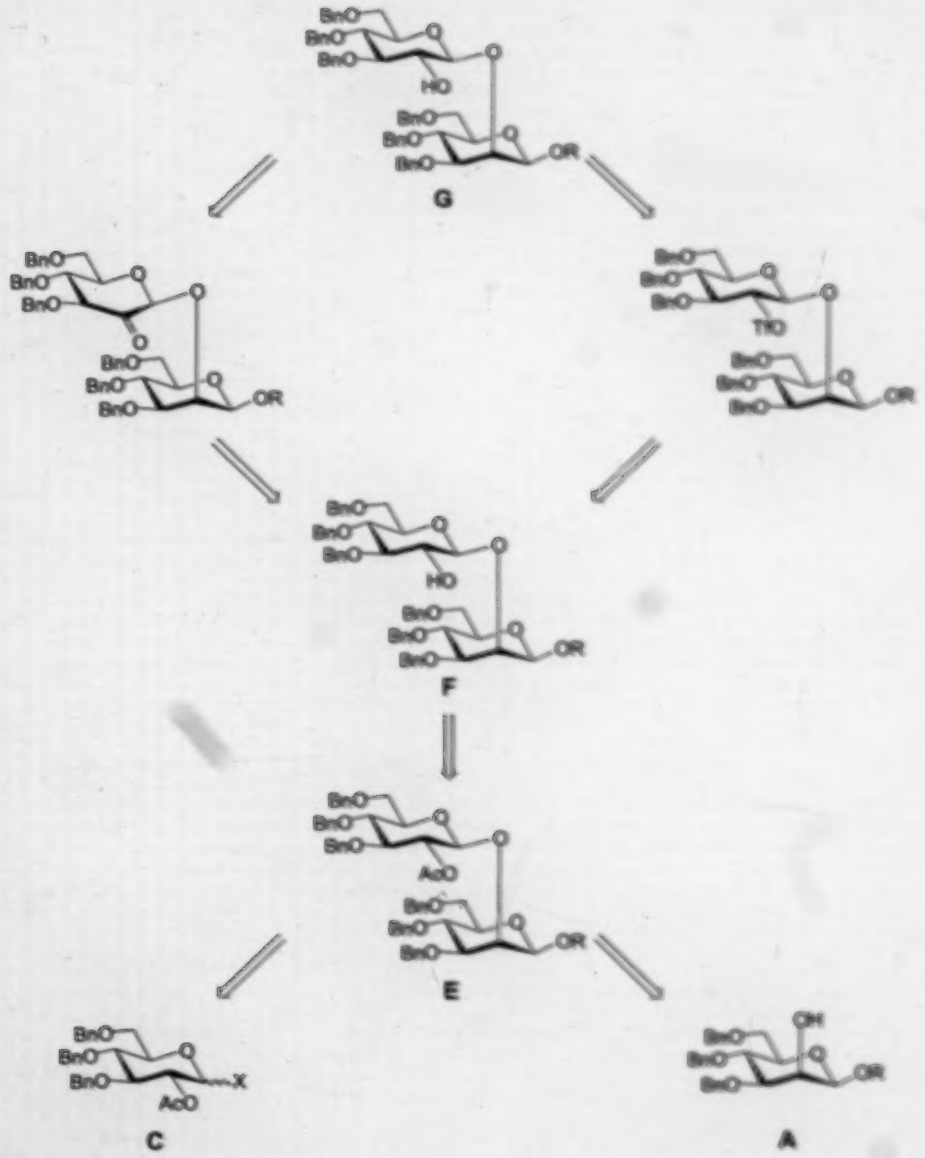


Figure 3.3 Retrosynthetic analysis of the β-(1→2)-mannopyranoside.

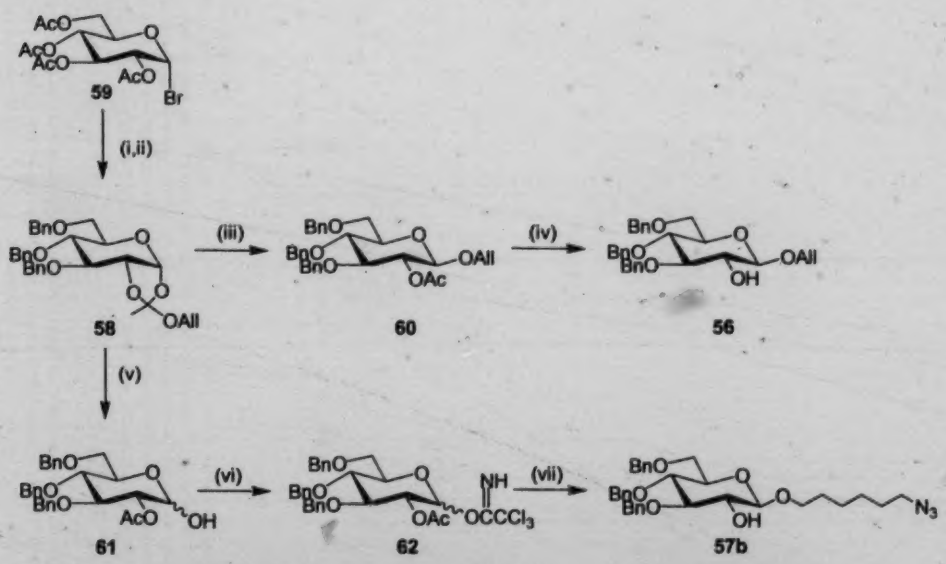
Acetates were installed as temporary O-2 protecting groups and benzyl ethers were used as persistent protecting groups for hydroxyl moieties not desired to react in the key synthetic steps. They can be easily installed, are

non-reactive with the reductive reagents used, and can be removed under catalytic or dissolving metal conditions at the end of the synthesis.

3.2b Synthesis of the Protected β-Mannopyranosyl Acceptor

Common building block **B** in Figure 3.2 is representative of the allyl 3,4,6-tri-*O*-benzyl-β-D-glucopyranoside **56** and azidohexyl 3,4,6-tri-*O*-benzyl-β-D-glucopyranoside **57b** intermediates (Scheme 3.7). Although **56** was previously made by Nitz and Wu, we have achieved a more efficient synthesis via direct conversion of the allyl orthoester to the corresponding glycoside. Orthoester **58** was cleanly obtained from the reaction of easily obtained 2,3,4,6-tetra-*O*-acetyl glucopyranosyl bromide **59** with allyl alcohol in the presence of 2,6-lutidine and TBABr in CH_2Cl_2 (83%). Simple Lewis acid promoted orthoester rearrangement of the 3,4,6-tri-*O*-benzyl-1,2-*O*-(*exo*-allyloxy)-ethylidene-α-D-glucopyranose **58** with $HgBr_2$ in CH_3NO_2 and toluene gave gram quantities of **60** in 76% yield and was then deacetylated under Zemplen conditions to afford **56**. Intermediate **58** also served as a precursor of **57**. Acid hydrolysis of **58** with 95% AcOH and pyridine gave **61**, which was isolated on a multi-gram scale by recrystallization from ethyl acetate and hexanes, thus eliminating a chromatographic purification step required in the previous synthesis. Subsequent transformation into the trichloroacetimidate donor **62** with CCl_3CN and DBU in CH_2Cl_2 was achieved in quantitative yield. Activation of **62**[78,125,126] with TMSOTf in CH_3CN in the presence of 6-azido-1-hexanol gave **57a** and further treatment with NaOMe

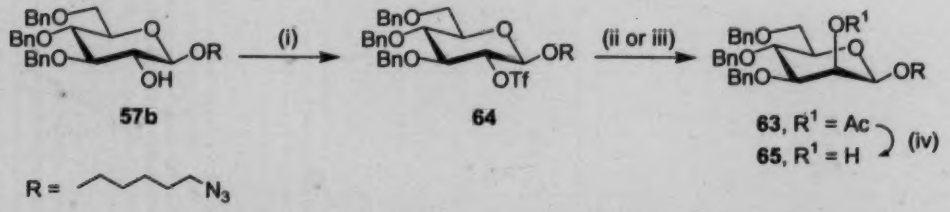
generated the deprotected azidohexyl β-D-glucopyranoside **57b** in 76% yield over the two steps.



Scheme 3.7 Formation of **56** and **57b**, precursors to β-mannopyranosyl acceptors. (i) 2,6-Lutidine, AllylOH, TBABr, CH_2Cl_2, 40 °C, 48 h, 83% (ii) BnBr, KOH, THF, 70 °C, 5h, 90% (iii) $Hg(Br)_2$, CH_3NO_2, toluene, 50 °C, 6.5h, 76% (iv) NaOMe, MeOH, RT, 40h, 89% (v) 95% AcOH, Pyridine, RT, 10h, 85% (vi) CCl_3CN, DBU, CH_2Cl_2, RT, 1h, quantitative (vii) a) 6-azido-1-hexanol, TMSOTf, CH_3CN, 0 °C, 30min, 85% (**57a**) b) NaOMe, MeOH, reflux, 1h, 90% (**57b**).

C-2 epimerization was performed on **57b** to form the 6-azidohexyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside **63** via the intermediate triflate **64** in 88% yield over two steps (Scheme 3.8). Triflation of **57b** with trifluoromethanesulfonyl anhydride in the presence of pyridine proceeded to give **64** cleanly. Inversion of the C-2 stereocenter was achieved with ultrasound assisted nucleophilic substitution. Subsequent ultrasonic S_N2 displacement with *n*-Bu_4NONO was first investigated as the corresponding

mannosyl alcohol **65** would be directly obtained; however, a poor yield (37%), degradation, and ring contraction products (16%) were found to complicate the reaction. The employment of *n*-Bu_4NOAc in toluene proved to be beneficial and allowed us to synthesize **63** in 88% yield on a gram scale without side products. This increased yield is in agreement with Fürstner's observations.[49] Epimerization of C-2 to produce β-D-mannopyranosides under these conditions is noteworthy as non-labour intensive steps are implemented and reaction progress is clear by thin layer chromatography (TLC).

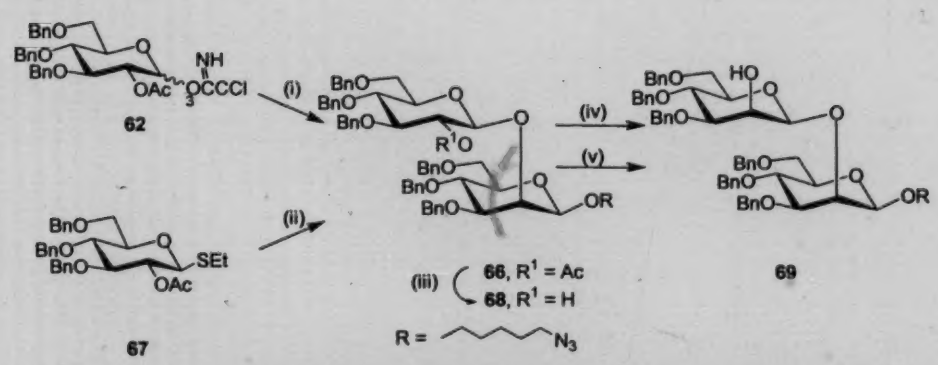


Scheme 3.8 Ultrasound promoted C-2 epimerization.(i) Tf_2O, pyridine, CH_2Cl, -40 °C to 10 °C, 4h (ii) *n*-Bu_4NONO, toluene, ultrasound, 12h, 37% 2 steps (iii) *n*-Bu_4NOAc, toluene, ultrasound, 12h, 88% 2 steps (iv) NaOMe, MeOH, reflux, 1h, 95%.

3.2c Synthesis of the Protected β-1,2-mannopyranoside

Extension of acceptor **65** with a β-D-glucopyranoside unit was attempted with two glucosyl donors (Scheme 3.9). Glycosylation with the previously made trichloroacetimidate donor **62** promoted with TMSOTf at 0 °C produced the desired compound **66** in low yield (42%) with many accompanying degradation compounds. Lowering the temperature, substituting CH_2Cl_2 with CH_3CN, and aliquot addition of the Lewis acid did

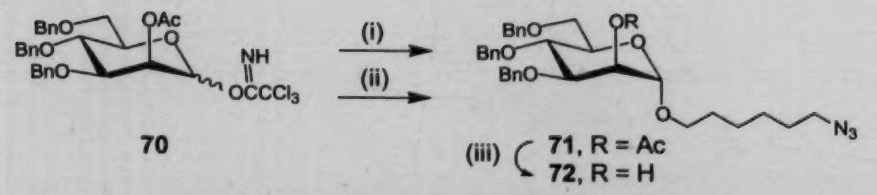
not increase the yield. A thioglycoside donor, ethyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-deoxy-1-thio-β-D-glucopyranoside **67**, was available for use from previous work by Henry Yu.[127] Activation of **67** with NIS and silver triflate produced the desired disaccharide **66** in good yield (81%). Transesterification of **66** gave the 6-azidohexyl 2-*O*-(3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside **68**. C-2 epimerization was first investigated using Fürstner's ultrasonic assisted displacement of triflate; however, the mannose epimer could not be isolated amongst the myriad of products. Due to time constraints, it is important to note that we did not proceed to study this reaction further; instead, a conventional oxidation-reduction sequence was used. The literature has examples of di- and pentasaccharides containing β-mannosyl units that have been prepared in this manner.[49,128] Oxidation of **68** with acetic anhydride and DMSO and sequential stereoselective reduction with L-Selectride in THF gave disaccharide **69** in good yield (80%).



Scheme 3.9 Extension of acceptor **65** with a β-D-glucopyranoside donors **62** and **67**. (i) **65**, TMSOTf, CH_2Cl_2, 0 °C to 10 °C, 45min, 42% (ii) **65**, NIS, AgOTf, -30 °C, 45min, 81% (iii) NaOMe, MeOH, reflux, 1h, 91% (iv) (a) Tf_2O, pyridine, CH_2Cl_2, -40 °C to 5 °C, 4.5h (b) *n*-Bu_4NOAc, toluene, ultrasound, 12h (v) (a) Ac_2O, DMSO, RT, 12h (b) L-Selectride, THF, -20 °C, 30min, 80% 2 steps.

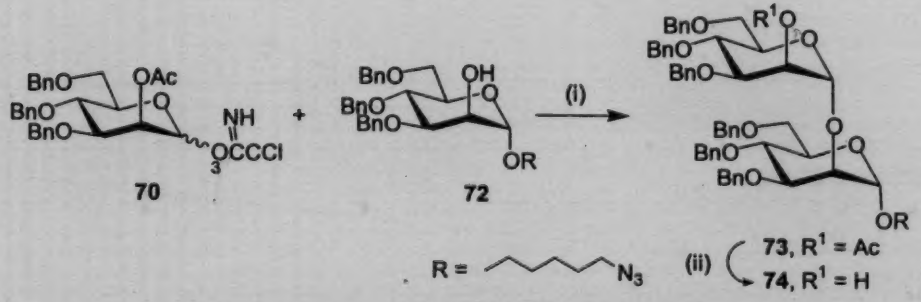
3.2d Synthesis of the Protected α-Glycosyl Acceptor and Elaboration to a Disaccharide and a Trisaccharide

The *trans* linkage found in α-(1→2)-mannopyranosides is easily formed by exploiting neighbouring group participation. Trichloroacetimidate donor **70** was easily obtained according to a published protocol.[51] Conjugation of **70** to 6-azido-1-hexanol was first attempted under TMSOTf activation in CH_2Cl_2 but resulted in a low yield of **71** (47%) (Scheme 3.10). Substitution of CH_2Cl_2 with CH_3CN increased the yield to 74%. Transesterification produced the reducing end acceptor **72**.



Scheme 3.10 Synthesis of the protected α-glycosyl acceptor **72**. (i) 6-azido-1-hexanol, TMSOTf, CH_2Cl_2, 0 °C to RT, 30min, 47% (ii) 6-azido-1-hexanol, TMSOTf, CH_3CN, 0 °C to RT, 1.5h, 74% (iii) NaOMe, MeOH, reflux, 1h, 96%.

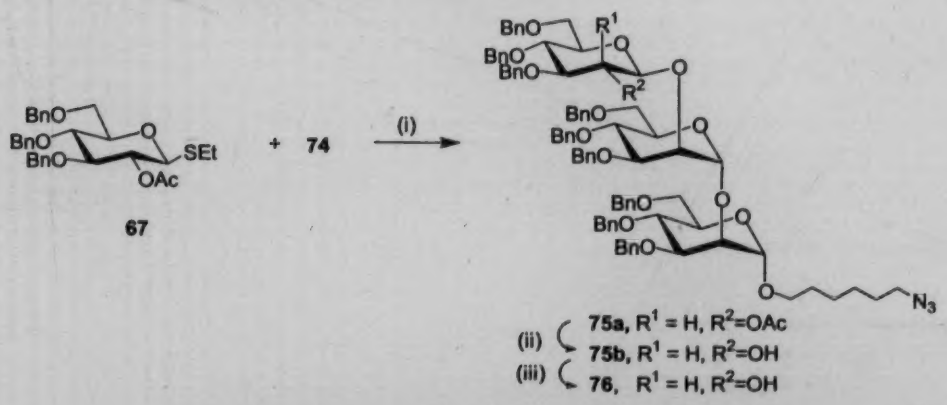
Elongation of the monosaccharide was carried out with donor **70** under the previously optimized activation conditions to give disaccharide **73** (Scheme 3.11). Subsequent saponification produced the deacetylated 6-azidohexyl 2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside **74**, which can be further used as the acceptor to form trisaccharide **75**.



Scheme 3.11 Preparation of disaccharide **74**. (i) TMSOTf, CH_3CN, 0 °C to RT, 55min, 87% (ii) NaOMe, MeOH, reflux, 1h, 73%.

Thioglycoside donor **67** was again used due to its success in adding a β-D-glucopyranosyl unit to an acceptor (Scheme 3.12). Activation of **67** with NIS and silver triflate and coupling to **74** produced the desired trisaccharide

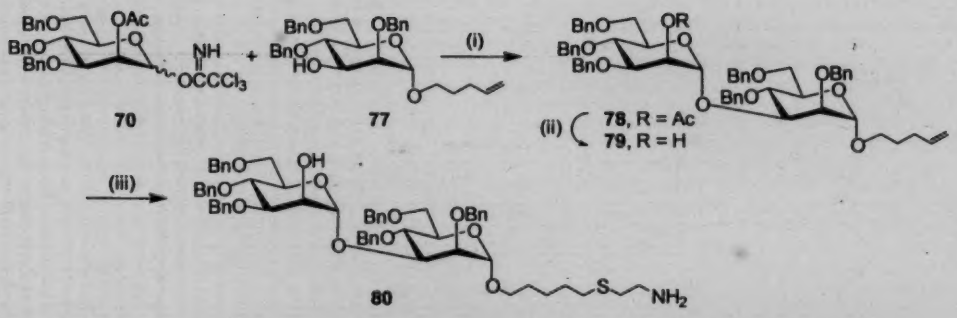
75 in good yield (85%). Sequential deacetylation, oxidation, and reduction under the same conditions used in the formation of the β-(1→2)-mannopyranoside formed trisaccharide **76**.



Scheme 3.12 Preparation of trisaccharide **76**. (i) (a) NIS, AgOTf, CH_2Cl_2, 0 °C to RT, 55min, 85% (ii) NaOMe, MeOH, reflux, 1h, 83% (iii) (a) Ac_2O, DMSO, RT, 12H (b) L-Selectride, THF, -20 °C, 30 min, 74% 2 steps.

3.2e Synthesis of the Protected α-(1→3)-Mannopyranoside

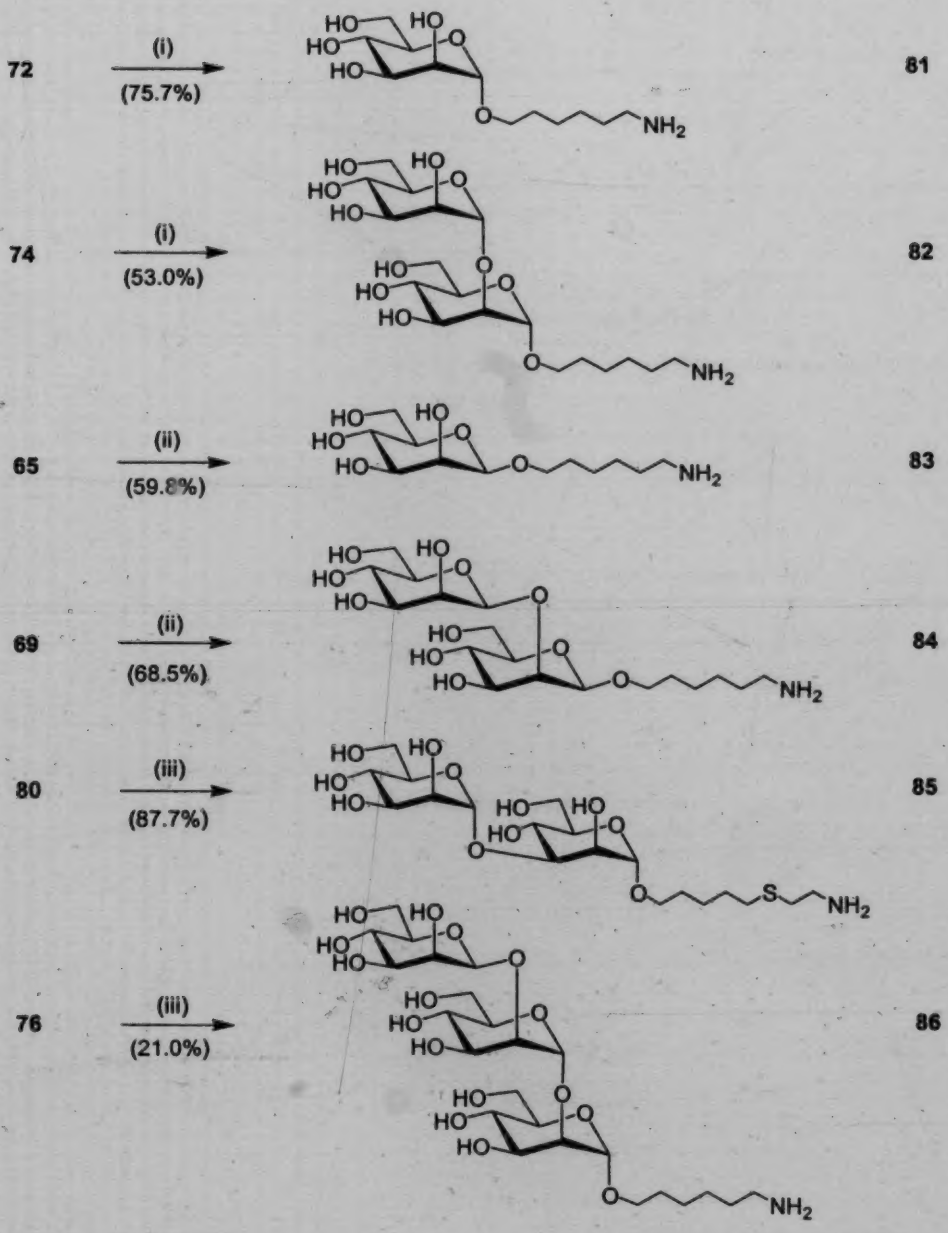
A suitably protected mannosyl alcohol **77** was available from previous work in this group and was used in our synthesis.[129] The presence of the pentenyl group did not pose an obstacle as the double bond could be derivatized with cysteamine to introduce an amine functionality (Scheme 3.13). Trichloroacetimidate donor **70** was combined with **77** under activation with TMSOTf in CH_3CN to afford **78**. Subsequent deacetylation gave disaccharide **79** in 91% yield over the two steps. Installation of the free amine was achieved by the radical photoaddition of 2-aminoethanethiol under long-wave ultraviolet light (365 nm) in a quartz vessel to give **80** in 80% yield.



Scheme 3.13 Synthesis of the protected α-(1→3)-mannopyranoside **80**. (i) TMSOTf, CH_3CN, -30 °C to 0 °C, 45min, not isolated (ii) NaOMe, MeOH, reflux, 1h, 91% 2 steps (iii) 2-aminoethanethiol hydrochloride, MeOH, CH_2Cl_2, *hv* 365nm, 12h, 80%.

3.2f Global Deprotection , Reduction of the azido–saccharides, and Conjugation to Fluorescein

Compounds **65**, **69**, **72**, **74**, and **76** must have their benzyl protecting groups removed and the azido functionality reduced to the free amine before conjugation to the fluorescent tag can be undertaken (Scheme 3.14). Deprotection of the α-mannopyranosides **72** and **74** was accomplished with palladium hydroxide,[52] otherwise known as Pearlman's catalyst, in methanol under one atmosphere of hydrogen gas to give products **81** and **82**. β-Mannopyranosides **65** and **69** were also deprotected under these conditions but with the addition of 2 or 3 drops of acetic acid respectively affording **83** and **84**. Deprotection of the α-(1→3)-mannoside **80** and trisaccharide **76** was achieved under dissolving metal conditions with sodium in liquid ammonia at -78 °C to give **85** and **86** in 88% and 21% yield correspondingly. The low yield of **76** may not be representative because we did not optimize the yield.



Scheme 3.14 The removal of benzyl protecting groups and the reduction of the azido functionality to the free amine. (i) $Pd(OH)_2$, $H_{2\,(gas)}$ (1 atm), MeOH, RT (ii) $Pd(OH)_2$, $H_{2\,(gas)}$ (1 atm), AcOH, MeOH, RT (iii) Na, NH_3, THF, MeOH, -78 °C.

Conjugation of the fluorescein-5-Ex-succinimidyl ester (Figure 3.4) to the free amines of **81-86** was performed under the same general conditions to give **87-92** (Scheme 3.15). A solution of the fluorescent reagent in CH_3CN was added to a triethylamine-methanol-water mixture of the specific aminoglycoside. The reaction mixtures were vortexed, centrifuged, and reacted for 2 hours. Excess fluorescent ester was removed by reverse-phase chromatography.

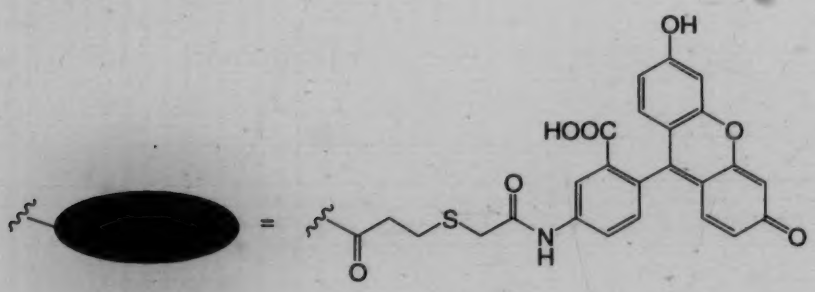
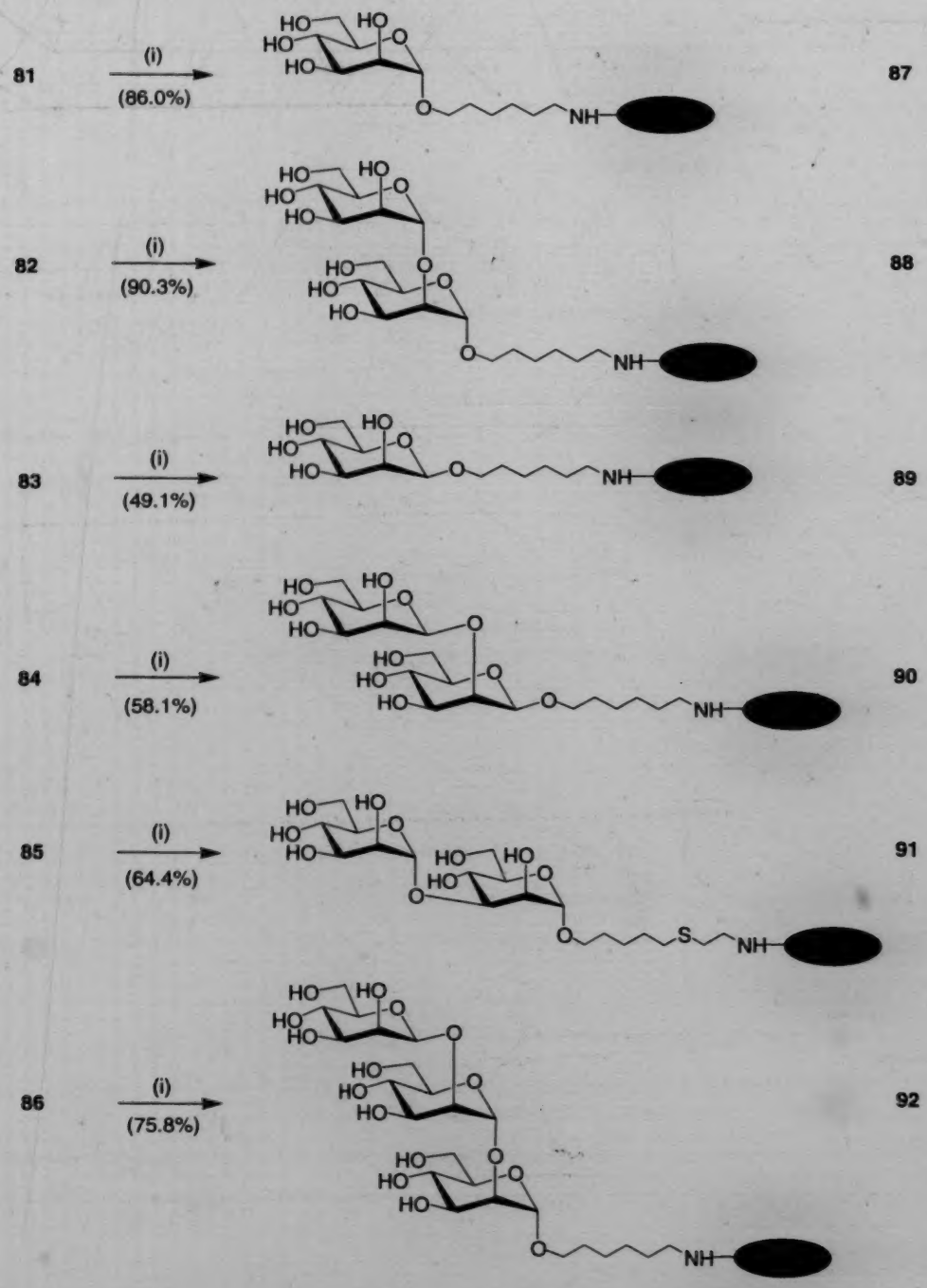


Figure 3.4 Representation of the fluorescein tag.



Scheme 3.15 Conjugation of the fluorescein-5-Ex-succinimidyl ester to the free amines. (i) Fluorescein 5-ex succinimidyl ester, Et_3N, MeOH, H_2O.

3.2g Summary and Further Work

The chemical synthesis of six acceptors **87-92** was achieved through novel and literature based protocols. Simple orthoester rearrangement in the installation of an allyl aglycon to form **60** proved to be successful and application of this strategy with 6-azido-1-hexanol is hypothesized to proceed well. The number of manipulations required to form acceptor **65** in the linear synthesis approach would therefore be reduced. Formation of the β-mannopyranosyl monosaccharide **89** was also optimized through the use of ultrasound promoted axial nucleophilic substitution of an equatorial triflate. The application of this methodology to the formation of β-(1→2)-mannopyranosyl oligosaccharides is of significant interest due to its ease of use. Therefore, more investigation is necessary to achieve success in this endeavour. Optimization of the removal of the protecting groups is required to increase the yield and availability of the deprotected compounds. For reasons to be discussed in the following chapter, choice of the linker chemistry between the sugar and the fluorescent group was important.

CHAPTER 4

Elucidation of Enzymatic Activity using Fungal Extracts from *Candida albicans*

4.1 Methods of Glycosyltransferase Identification

Identification of the glycosyltransferases responsible for building the mannopyranose chains found in the cell wall of *Candida albicans* is possible through a two pronged strategy. First, sequence homology of the annotated genome has identified nine families of glycosyltransferase genes in *C. albicans*. Expression of candidate mannopyranosyltransferase genes combined with activity assays should lead to the determination of their function. Second, a variety of extracted mannan acceptors can be conjugated to a fluorescent moiety and employed to probe crude fungal extracts. Enzyme activity is usually followed by chromatography and the products are evaluated through analytical methods. Extract fractions enriched in glycosyltransferase activity can then be subjected to a proteomics based analysis to identify the proteins present and eventually facilitate the identification of transferases in the genome annotation.

4.1a *Candida albicans* Mannopyranosyltransferases in Literature

Although many studies concerning the synthesis of yeast cell wall mannoproteins have been done in *Saccharomyces cerevisiae*, only α-(1→2)- and α-(1→6)-mannopyranosyltransferases have been identified in the *Candida albicans* genome to date.[130,131] β-(1→2)-mannopyranosyltransferase activity has been detected in a crude fungal extract; however, the gene sequence of these enzymes has not yet been elucidated.[132] The enzymes mentioned in the following discussion were obtained through the two strategies described above, respectively.

A DNA sequence homologous to a mannosyltransferase gene in *Saccharomyces cerevisiae* was identified through various restriction digest and hybridization experiments in the *Candida albicans* genome and named *CaMNT1*.[130] Over-expression of the gene product, CaMNT1p, in *Pichia pastoris* resulted in high levels of mannosyltransferase activity. Subsequent gene disruption demonstrated a large reduction of *in vitro* activity reflected by the production of truncated *O*-linked mannan structures. Wild type CAI4 and homozygous CaMNT1 disrupted NGY24 *C. albicans* strains were incubated with tritiated mannopyranose. Isolation of the *O*-linked oligosaccharides and separation by high-performance thin-layer chromatography (HPTLC) indicated that CAMNT1p is responsible for adding the second mannose residue to *O*-linked oligosaccharides (Figure 4.1 Lane 1-2). Analysis of the wild type oligosaccharides by an α-(1→2)-mannosidase digest revealed that

CAMNT1p is in fact an α-(1→2)-mannopyranosyltransferase (Figure 4.1 Lane 3-5).[130]

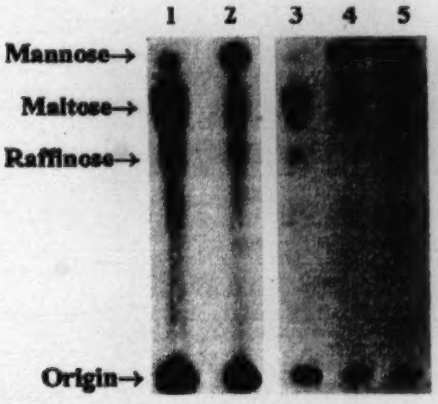


Figure 4.1 Autoradiogram of an HPTLC chromatogram of β-eliminated [^{3}H]mannose-labeled mannan isolated from *C. albicans* CAI4 (lane 1) and the derived homozygous disruptant strain, NGY24 (lane 2). In a separate experiment, β-eliminated mannan isolated from *C. albicans* CAI4 was left undigested (lane 3) or digested with jack bean α-mannosidase (lane 5) or α-(1→2)-mannosidase of *A. satoi* (lane 4) prior to chromatography. Taken from E.T. Buurman *et al.*[130]

The substrate specificity of CaMNT1p was probed with a variety of α-linked mono-, di-, tri-, penta- and hexa-mannopyranosyl oligosaccharides (Figure 4.2).[133] The methyl α-D-mannopyranoside was the most efficient acceptor and hence supports the earlier observation that this transferase is responsible for adding the second mannose residue to *O*-linked oligosaccharides. CaMNT1p also showed high activity with α-(1→2)-mannobiose and therefore could also play a role in adding a third mannpyranosyl residue to the growing oligosaccharide chain. Effective transfer to the *N*-linked glycan core structure, $GlcNAcMan_5$, also occurred *in*

vitro. However, it is unlikely, that this type II membrane-bound Golgi enzyme is involved in the assembly or subsequent elaboration of the N-glycan core which occurs in the endoplasmic reticulum. Yet, CaMNT1p may be able to participate in N-glycosylation *in vivo* in the extension of the α-(1→2)-mannopyranose outer chain branches. The decreased level of activity seen with the remaining acceptors indicates the preference of CaMNT1p for α-(1→2)-mannopyranosyl substrates.[133] Accommodation of a range of acceptors is not unusual for glycosyltransferases, however their linkage specificity is more stringent – "one enzyme, one linkage".

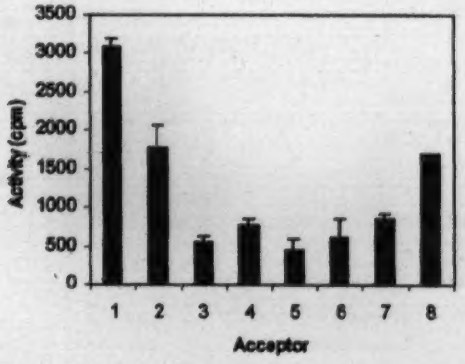


Figure 4.2 Mannopyranosyltransferase activity of CaMnt1p using a range of oligosaccharide acceptors: methyl α-D-pyranoside (*lane 1*), α-(1→2)-D-mannobiose (*lane 2*), α-(1→3)-D-mannobiose (*lane 3*), α-(1→4)-mannobiose (*lane 4*), α-(1→6)-D-mannobiose (*lane 5*), α-(1→3)-α-(1→6)-D-mannotriose (*lane 6*), α-(1→3)-α-(1→6)-α-(1→3)-α-(1→6)-D-mannopentose (*lane 7*), and Man5GlcNAc-hexasaccharide (*lane 8*). Taken from L.M. Thomson *et al.*[133]

Disruption of the *CaMNT1* gene results in a reduction in fungal adherence and virulence.[130] The absence of CaMNT1p led to a 95% decrease

in adherence of *Candida albicans* to buccal epithelial cells. In a vaginal candidiasis model, rats injected with approximately 10^6 and 10^7 colony forming units (CFUs) of wild type SC5314 *C. albicans* showed the presence of 40 to 200 fungal CFUs in vaginal lavages at subsequent time points. However, rats injected with heterozygous and homozygous disrupted *CaMNT1* strains NGY21 and NGY23 respectively, completely cleared CFUs after 3 weeks. In another experiment, the virulence of systemic candidiasis was evaluated with SC5314, NGY21, and NGY23 strains of *C. albicans* in immuno-competent guinea pigs. A decreased mortality rate was observed with the disrupted fungal strains (Figure 4.3). Internal organs were analyzed for infection and increased numbers of non-colonized liver, kidney, and skin were found in guinea pigs intravenously infected with the three strains of *C. albicans* (Table 4.1). Similar numbers of C. *albicans* cells recovered from positive tissues from all three strains indicate that attenuated virulence results from the reduced ability of the fungal cells to reach and adhere to various organs. However, invasion and proliferation of the fungus is not impeded once the organ is successfully colonized.[130]

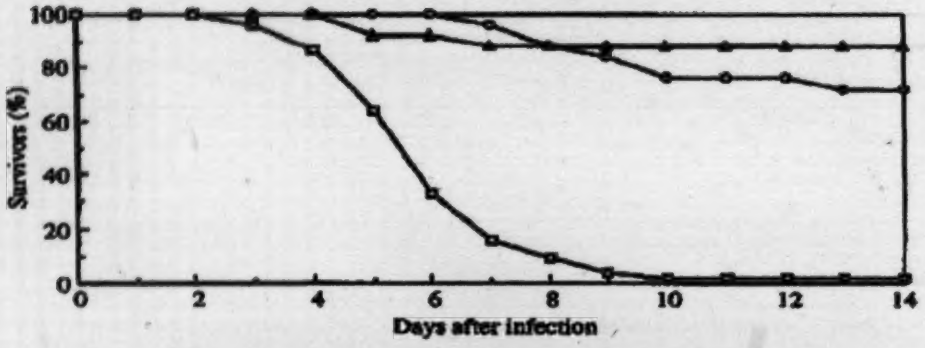


Figure 4.3 Survival of Pirbright guinea pigs (500 g) intravenously infected with 40000 CFUyg of *C. albicans* SC5314 (□) and prototrophic heterozygous (○) and homozygous *CaMNT1* (▲) disruptant strains, NGY21 and NGY23 strains respectively. Taken from E.T. Buurman *et al.*[130]

Table 4.1 Colonization of various sites (in log CFUyg 6 1.0) in guinea pigs (40,000 CFU/g) after i.v. infection with *C. albicans* SC5314 and derived prototrophic heterozygous and homozygous *CaMNT1* disruptant strains NGY21 and NGY23, respectively. Taken from E.T. Buurman *et al.*[130]

			Negative organs, %			Colonization organs		
Strain	*n*	Survivors, %	Liver	Kidney	Skin	Liver	Kidney	Skin
Guinea pig								
SC5314	45	2	36	0	7	3.5	5.4	4.2
NGY21	25	72	76	60	32	3.5	3.9	3.8
NGY23	25	88	88	88	60	3.4	4.8	3.6

Against all expectation, homozygous knock-out of the *CaMNT1* gene did not completely disrupt *O*-linked glycosylation as 25% of specific *in vitro* mannosyltransferase activity remained.[130] Therefore, another enzyme was suggested to be functionally redundant as has been seen in *Saccharomyces cerevisiae.* A second member of the *CaMNT* gene family, *CaMNT2*, was identified through homologous hybridization experiments.[134] Disruption of *CaMNT2* led to the truncation of the *O*-linked mannpyranosyl chain after the

second mannopyranose residue hence suggesting that CaMNT2p predominantly adds the third sugar unit but plays a minor role in adding the second residue. A double *Camnt1-Camnt2* mutant was significantly less virulent then either single gene mutant due to the inhibition of cell wall *O*-glycosylation.[134]

A second mannosyltransferase, an α-(1→6)-mannopyranosyltransferase, responsible for the synthesis of the α-(1→6)-linked side chain branching units (Figure 2.5) was identified by Suzuki and coworkers.[131] A mannan pentaose, α-Man-(1→3)-α-Man-(1→2)-α-Man(1→2)-α-Man-(1→2)-Man, was isolated from the *Candida albicans* cell wall phosphomannan through mild acetolysis and further pyridylaminated.[103] Incubation of this substrate with a particulate detergent insoluble fraction from the fungal extract of *Candida albicans* NIH B-792 (serotype B) and α-GDP-mannose gave a single pyridylamino-manno-hexaose product by HPLC. The linkage sequence and branch point were determined to be [α-Man-(1→3)-α-Man-(1→6)]-α-Man(1→2)-α-Man(1→2)-α-Man(1→2)-Man through a combination of an ELISA assay and two-dimensional homonuclear Hartman-Hahn Nuclear Magnetic Resonance (HOHAHA NMR) spectroscopy.[131] Substrate specificity of the α-(1→6)-mannopyranosyltransferase was probed with various pyridylaminated mannan oligosaccharides (Table 4.2).[131] Incubation of these acceptors with a *C. albicans* fungal extract and subsequent analysis determined that the α-(1→6)-mannopyranosyltransferase required the presence of an α-(1→3)-linked mannose in the substrate, preferably at the

non-reducing terminus. Furthermore, the linkage of the third mannose unit from the terminal end was not critical to the transferase activity.[131]

Table 4.2 Substrate specificity of the α-(1→6)-mannopyranosyltransferase. Taken from Suzuki *et al.*[131]

structure	Mannose incorporation by *C. albicans* NIH B-792
	nmol · μg protein^{-} · h^{-1}
Manα1−3Manα1−6Manβ1−4GlcNAcβ1−4GlcNAc-pyridylamine	289
Manα1−3Manα1−2Man-pyridylamine	493
Manα1−2Manα1−2Manα1−2Man-pyridylamine	0
Manα1−3Manα1−2Manα1−2Man-pyridylamine	165
Manα1−3Manα1−2Manα1−2Manα1−2Man-pyridylamine	145
Manα1−3Manα1−3Manα1−2Manα1−2Man-pyridylamine	486
Manα1−2Manα1−3Manα1−2Manα1−2Manα1−2Man-pyridylamine	14
Manα1−3Manα1−2Manα1−3Manα1−2Manα1−2Man-pyridylamine	96
Manα1−3Manα1−2Manα1−2Manα1−2Man-pyridylamine ↑6 Manα	33

Lastly, a β-(1→2)-mannopyranosyltransferase has been detected in the crude fungal extract of *Candida albicans* J-1012 (serotype A) and NIH B-792 (serotype B) strains.[132] The biosynthetic pathway of serotype A specific β-mannan epitopes (Figure 4.4) suggested that detection of the β-(1→2)-mannopyranosyltransferase II (β-1,2-MNT II) would be more facile than the β-1,2-MNT I as the latter enzyme competes with the α-(1→3)-mannopyranosyltransferase for the common intermediate α-Man_4. Therefore, αβ-Man_5 was used as substrate to probe the fungal extract for addition of a second β-mannosyl unit. Incubation of αβ-Man_5 with a particulate detergent insoluble fraction from the fungal extract of *Candida albicans* J-1012 and GDP-mannose produced a single mannohexapyranose (Figure 4.4). The enzyme reaction product was determined to contain a terminal β-linkage due

to its inertness to digestion by *exo*-α-mannosidase and its structure was confirmed to be αβ-Man_6 by ^{1}H-NMR.[132]

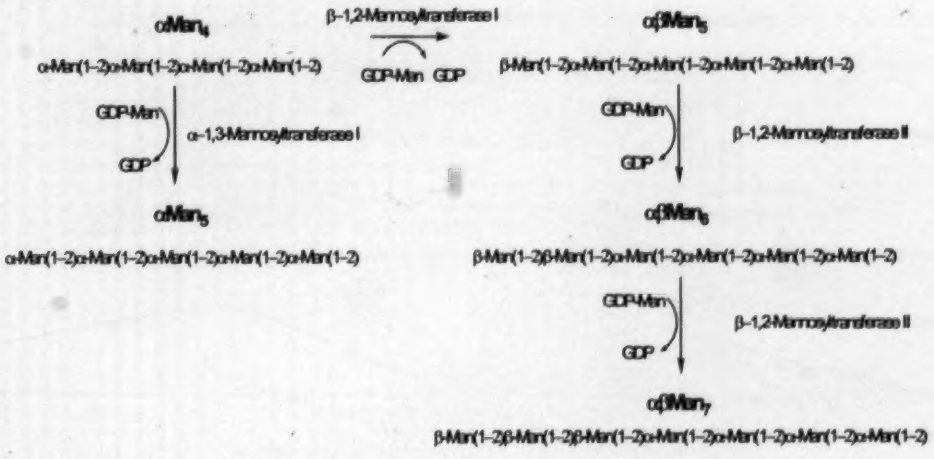


Figure 4.4 The biosynthetic process of mannan side chains in serotype A *Candida albicans* strains. Taken from Suzuki *et al.*[132]

The substrate specificity of β-1,2-MNT II was assessed with a variety of pyridylaminated oligosaccharides (Table 4.3).[132] Moderate transferase activity was observed with PA-αβ-Man_3, PA-αβ-Man_5, and PA-αβ-$SMan_5$; however, none was seen with PA-α-Man_4, PA-αβ-Man_6, or any of the β-(1→2)-linked oligomers. These results indicate that the enzyme requires an α-(1→2)- or α-(1→3)-mannopyranosyl residue to precede the terminally linked β-(1→2)-mannose to form an active substrate.[132]

Table 4.3[132] Substrate specificity of the β-1,2-mannopyranosyltransferase II activity. *Relative activity (%). Blue box highlights positive results and the red box highlights an unexpected result. Taken from Suzuki *et al.*[132]

Abbreviation	Structure	Mannose incorporated (nmol/mg protein/h)	
		C. albicans J-1012	*C. albicans* NIH B-792
PA-βMan_2	Manβ1-2Man-PA	0 (0)*	
PA-βMan_3	Manβ1-2Manβ1-2Man-PA	0 (0)	–
PA-$\alpha\beta Man_3$	Manβ1-2Manα1-2Man-PA	20 (69)	
PA-βMan_4	Manβ1-2Manβ1-2Manβ1-2Man-PA	0 (0)	–
PA-αMan_4	Manα1-2Manα1-2Manα1-2Man-PA	0 (0)	–
PA-$\alpha\beta Man_5$	Manβ1-2Manα1-2Manα1-2Manα1-2Man-PA	29 (100)	56 (193)
PA-$\alpha\beta Man_5$	Manβ1-2Manα1-3Manα1-2Manα1-2Man-PA	29 (100)	–
PA-$\alpha\beta Man_6$	Manβ1-2Manβ1-2Manα1-2Manα1-2Manα1-2Man-PA	0 (0)	

Unexpectedly, incubation of PA-αβ-Man_5 with the particulate detergent-insoluble fraction of the fungal extract of *Candida albicans* NIH B-792 and GDP-mannose produced αβ-Man_6.[132] This result was unforseen as this serotype B strain does not contain this serotype A specific antigen. It was therefore postulated that the absence of serotype A epitopes in serotype B strains is due to the mutation or absence of the initial key β-1,2-MNT I. Hence, activity of β-1,2-MNT I is the point of divergence between the serotypes observed in *C. albicans*.[132]

4.2 Enzymatic Reaction Product Detection and Analysis

The progress of enzymatic reactions can be easily monitored by TLC, matrix-assisted laser desorption (MALDI) mass spectrometry, and capillary electrophoresis (CE). The introduction of a new carbohydrate residue is detected as a decrease in polarity in TLC, an increase in mass per charge in MALDI, and a change in migration time in CE. A brief introduction to CE is presented in section 4.2a. The nature of the glycosidic linkage, α or β, can be

determined through specific glycosyl hydrolase digestions with α- and β-*exo*-mannosidases and the substituted position on the parent carbohydrate ring can be determined with the collection of a one- and two-dimensional (1D and 2D) NMR experiments. For example, heteronuclear multiple bond correlation (HMBC) experiments identify proton nuclei with carbon nuclei that are separated over two or three bonds. Hence, correlation across the glycosidic bond C_1'-O-C_x-H_x can indicate the bonding position between adjoining carbohydrate units.

4.2a Capillary Electrophoresis (CE)

CE is a high performance analytical technique based on the differential electrophoretic migration of analytes.[135] Many forms of CE exist; however, capillary zone electrophoresis (CZE) is the simplest and most encountered form of CE. CZE relies on the charge-to-mass ratio of the solutes for separation (Figure 4.5). Constant electrical field strength inside a fused silica capillary is crucial to this method and is generated by an external high voltage (10-30kV) power supply. The field is maintained by the presence of a background electrolyte, a homogeneous aqueous buffer. Analytes are therefore separated according to their attraction to the anode (positive) or cathode (negative) and solute peaks are then analysed by a detector as they exit the capillary.[135]

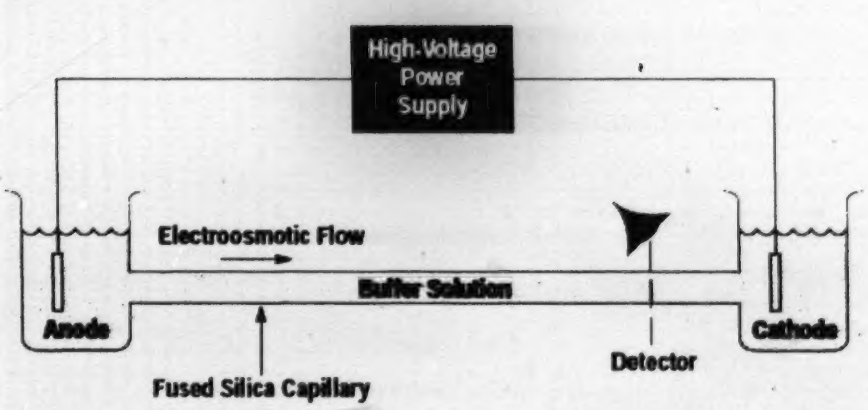


Figure 4.5 A schematic of capillary zone electrophoresis. Taken from R. R Holloway.[136]

Many compounds have an inherent ability to ionize due to the pH controlled dissociation of acidic groups or protonation of basic groups. Functionalities such as aromatic groups that are commonly found in organic molecules also impart chromophoric qualities to the analyte, which is fundamental for detection. However, carbohydrates generally lack readily ionisable functional groups or chromophores and hence must be modified with a reagent such as 1-aminopyrene-3,6,8-trisulfonate (APTS) to provide sufficient charge and strong fluorescence. A running buffer, such as borate, can also chelate with the hydroxyl groups of the carbohydrate moiety to impart an overall negative charge and also form a low ultra-violet (UV) active complex.[137]

Sample injection into the capillary occurs hydrodynamically or electrokinetically.[135] Hydrodynamic insertion is accomplished with either a timed over-pressure on the input end or under-pressure on the output end of

the capillary. Loading of a sample by electrokinetic injection requires the application of an injection voltage for a short time and is dependent on the electromigration of each analyte. However, the latter method is generally less reproducible.[135]

Migration of analytes in CZE is mainly affected by the electroosmotic flow (EOF).[136] The inner surface of the capillaries is coated with negatively charged dissociated silanol groups produced when fused silica is in an aqueous medium (Figure 4.6).[135] Hydronium ions and other cations remain localized along the wall to neutralize the charge (Figure 4.7). When a voltage is applied across the capillary this positively charged cylinder is attracted to the cathode and trapped water molecules co-migrate to produce a net flow of buffer solution in the direction of the negative electrode. However, the movement of the positive column does not agitate the analyte bands due to its flat-profile flow.[136] Diffusion is also minimized due to short experimental times; therefore resolution of closely related compounds, such as diastereomers, can be achieved.[135]

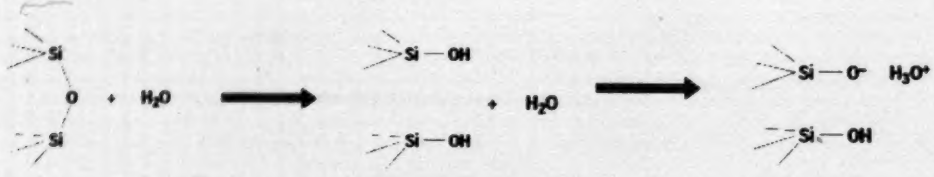


Figure 4.6 Hydration of a raw silica surface to polysilic acid and subsequent charging of silica by ionization of the surface in an aqueous medium. Taken from R. R Holloway.[136]

The velocity of the EOF is directly proportional to the voltage applied across the capillary; the higher the voltage, the faster the flow. Hence separation of the analytes can be optimized with changes in voltage levels.[135]

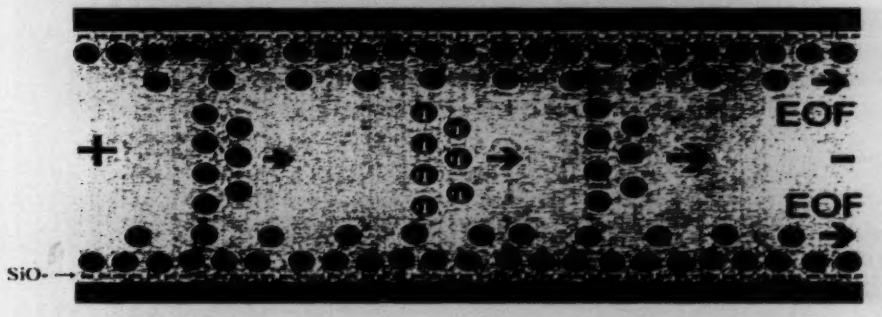


Figure 4.7 The EOF and the resulting net flow of all charged particles toward the cathode.

Compound detection is usually dependent on the absorbance or fluorescence of an analyte band passing through a detector. UV or laser-induced fluorescence (LIF) are the most common modes of detection; although, indirect methods, such as CE coupled mass spectrometry, are possible for analytes that do not contain sufficient chromophore activity in the UV-Visible light region (180-800 nm). LIF is typically 500 times more sensitive for compounds that fluoresce than UV detection and thus has attracted much attention due to this high sensitivity.

Detector response of the analytes yields a characteristic peak profile termed an electropherogram (Figure 4.8). Qualitative peak characteristics are related to migration time (MT) and the area under the curve is proportional to their concentration. A comparison of the peaks present gives an indication of

the relative abundance of each compound in the sample. Therefore, in monitoring the progress of enzymatic reactions, CE provides the vital percent conversion of substrate to product. The relational placement of the peaks is dependent on each analyte.

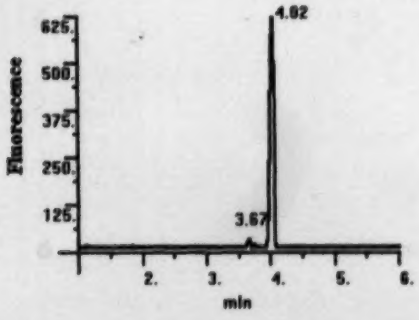


Figure 4.8 CE/LIF electropherogram for fluorescein (MT=4.02 min) standard at 1.0 X 10^{-7} M; internal standard erythrosin B (MT=3.67 min). Taken from W.C. Brumley *et al.*[138]

4.3 Scope of Project

Although several mannopyranosyltransferases from *Candida albicans* have been previously characterized, our work was directed towards the identification of the β-(1→2)-mannopyranosyltransferases responsible for generating antigenic factors 5 and 6. Due to the difficulty associated with the chemical synthesis of the conjugate vaccine carbohydrate epitopes, fluoreceinated mannan acceptors **81-86** were employed to probe crude fungal extracts of *C. albicans* for β-mannopyranosyltransferase activity. Enzymatic assays and subsequent analyses of the reaction products were performed.

4.4 Overview

In the pursuit of a conjugate vaccine against *Candida albicans*, the native glycosyltransferases that produce the highly immunogenic β-mannan was of interest. Cells of the CAI4 serotype A strain were obtained and homogenized. A detergent insoluble fraction from the fungal extract was isolated and incubated with acceptors **87-92**. Initial tests with all acceptors and subsequent optimization assays with **89**, **90**, and **91** revealed the presence of mannosyltransferase activity. Determination of the addition of a mannopyranose unit was accomplished using a CE-LIF detection system with an argon laser. Extension of the parent residue always appeared as a new peak to the left of the substrate. Large enzymatic assays were done with acceptors **89**, **90**, and **91** to generate analytical quantities of product to more specifically probe the nature of the linkage between the new mannose unit and the parent chain. MALDI was used to determine the number of sugar residues added to the substrate in these scaled up reactions. The new linkages were first classified as α or β using α- and β-mannosidase digestion assays. The specific point of attachment of the new residue was elucidated from one- and two-dimensional NMR experiments.

4.4a CAI4 *Candida albicans* Cell Growth and Crude Extract Preparation

CAI4 *Candida albicans* from a glycerol stock were streaked on YPD plates. After overnight incubation at 30 °C, a single colony of the fungi was

inoculated into YPD media. The culture was incubated overnight and the cells harvested by centrifugation. The cell pellet was subsequently frozen at -70 °C.

The cells were resuspended in 50mM HEPES, pH 7.0, containing 10% glycerol and lysed. Low speed centrifugation (5000 rpm) followed by filtration of the supernatant allowed the removal of cellular debris. After ultracentrifugation at 45,000 rpm, the supernatant was recovered and put aside (fraction S), whereas the pellet was resuspended in 50mM HEPES, pH 7.0 (fraction P) and solubilized in 0.2% POE. Further ultracentrifugation at 45,000 rpm and resuspension of the pellet afforded the crude enzyme extract fraction POE-P.

4.4b General Fungal Extract Assay with Acceptors 87-92

Transferase activity of membrane fractions S and POE-P was assayed by incubation with fluorescein-labeled mannan acceptors **81-86** and α-GDP-mannose. General assay conditions, HEPES buffer at a biological pH (7.0), $MnCl_2$, $MgCl_2$, and dithiothreitol (DTT) were employed in the initial acceptor screening. The divalent metal ions were used because many enzymes require them as cofactors. DTT, at low concentrations, stabilizes enzymes that possess free sulfhydryl groups and prevents the loss of activity due to oxidation of these groups *in vitro*. The reaction products were evaluated by TLC and CE however their structure was not determined. Although both fractions gave the same products, their distribution varied with **87**, and a general increase in

substrate conversion was observed for **88, 89, 91,** and **92**. Acceptor **90** did not indicate any elaboration (Table 4.4).

Table 4.4 Transferase activity of membrane fractions S and P with acceptors **87-92** measured by CE in Tris-Borate-EDTA (TBE) buffer pH 8.28 and * Tris-Borate-EDTA with 20mM SDS (TBES) buffer pH 8.28.

Acceptor	Extract Fraction	Product 1 (% conversion)	Product 2 (% conversion)	Time (min)
87	S	72.50	9.62	30
	POE-P	48.52	51.04	30
88	S	28.85	-	30
	POE-P	73.23	-	30
89	S	2.08	-	90
	POE-P	4.64	-	90
90	S	No Reaction		90
	POE-P	No Reaction		90
91	S	CE Inconclusive		30
	POE-P	CE Inconclusive		30
92	S	5.86*	-	90
	POE-P	17.19*	-	90

4.4c Optimization of the Fungal Extract Assay with Acceptor 89

Attempts to increase the conversion of substrate **89** to product were successful with various solubilization conditions of the fungal extract fractions and longer reaction times. Increased reaction times were hypothesized to increase the conversion of substrate to product. Remarkably the enzymes were found to remain active until the seventh day. The effect of detergent for fractions S and P was monitored over a period of seven days (Figure 4.9). Expectedly, the absence of a detergent with fraction S led to poor conversion

of the substrate; however, with fraction P, conversion of **89** to product was achieved at the same level as the solubilized fractions. The 0.5% POE detergent pellet fraction was observed to dramatically increase in activity after the third day and reached 39.85% percent conversion after seven days. The behaviour of this particular reaction does not correlate with the others for an unknown reason.

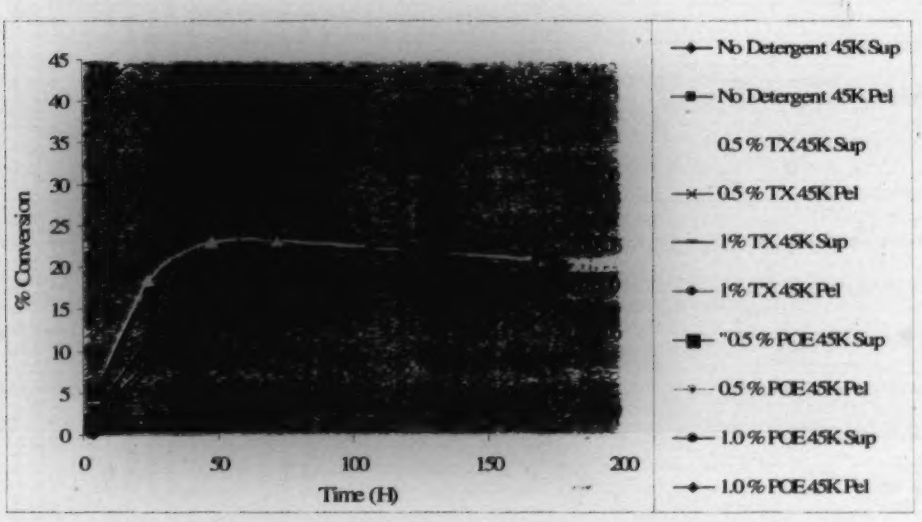

% Conversion
Time (H)
No Detergent 45K Sup
No Detergent 45K Pel
0.5 % TX 45K Sup
0.5 % TX 45K Pel
1% TX 45K Sup
1% TX 45K Pel
"0.5 % POE 45K Sup
0.5 % POE 45K Pel
1.0 % POE 45K Sup
1.0 % POE 45K Pel

Figure 4.9 Effects of various solubilization conditions of fraction P on conversion of **89** to product.

4.4d Optimization of the Fungal Extract Assay with Acceptor 90

The conversion of substrate **90** to one product was achieved by extending the reaction time from 90 min to 2 days (Table 4.5). The similar conversion activity between assays 1 and 2 indicates that the use of DTT is neither beneficial nor harmful in these small scale reactions. The very low activity seen in assay 3 with magnesium in comparison with assay 4 clearly

demonstrates the enzyme's requirement for manganese. The additional presence of magnesium in assays 5-7, however, does not affect the enzymatic activity negatively. From this initial optimization, the transferase showed a preference for pH 6.0.

Table 4.5 Optimization of the fungal extract assay with acceptor **90**.

Assay	Ion 1	Ion 2	pH	DTT	% Conversion Day 1	% Conversion Day 2
1	Mn	-	6.0	DTT	3.14	5.62
2	Mn	-	6.0	-	3.37	6.61
3	-	Mg	7.0	DTT	0.21	-
4	Mn	-	7.0	DTT	3.23	-
5	Mn	Mg	6.0	DTT	4.25	11.47
6	Mn	Mg	7.0	-	3.38	7.14
7	Mn	Mg	8.0	DTT	0.62	-

The pH dependency of the enzyme was determined in HEPES and ammonium acetate (AA) buffer between pH 4.5 and 7.0 (Figure 4.10). After two days, maximum activity was obtained with AA at pH 6.0. Extrapolation of the data for pH 6.0 indicated that this condition would give the most product after four days.

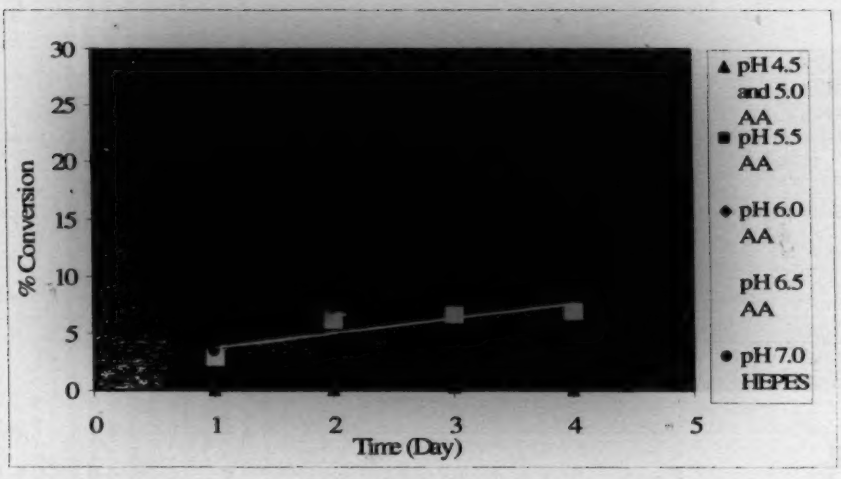


Figure 4.10 Effect of pH on product formation with acceptor **90**.

The use of detergents was investigated with acceptor **90**; however no significant effect could be detected by CE. Therefore, fungal extract fraction P was used in all the following assays. Substrate conversion was also found to be dependant on the initial concentration of α-GDP-mannose (Table 4.6, Figure 4.11). Four equivalents of donor to acceptor increased the formation of product 1.5 fold. The increased amount of product was subsequently transformed or hydrolyzed after day four and eventually returned to the level observed with the control assay at 1.0M α-GDP-mannose on day eight. The long reaction times were again determined by trial. A large excess of α-GDP-mannose could not be used as the accumulation of the di-phosphate by-product inhibits the transferase reaction. The future use of an acid phosphatase could relieve this concern.

Table 4.6 Effect of α-GDP-mannose concentration on product formation.

Day	% Conversion	
	1.0M α-GDP Mannose	2.0M α-GDP Mannose
1	4.76	10.64
2	5.91	10.17
4	12.59	18.78
7	14.33	17.74
8	14.41	15.28

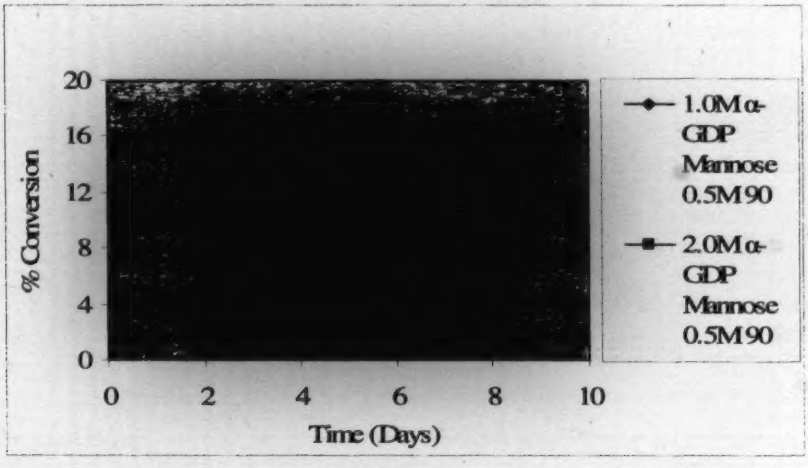


Figure 4.11 The effect of α-GDP-mannose concentration on product formation.

4.4e Optimization of the Fungal Extract Assay with Acceptor 91

CE analysis of the initial fungal extract screen with **91** showed the presence of three large peaks that were attributed to starting material related analogues and five smaller product peaks (Figure 4.12). The pre-reaction substrate distribution was very similar and was unanticipated from the ^{1}H NMR of disaccharide **91**. Each starting material peak was isolated by reverse

phase HPLC and subsequent analysis by MALDI revealed varying levels of oxidation of the sulphur atoms in the alkyl chain linking the carbohydrate to the fluorescein molecule (Figure 4.13). The large amount of oxidation for **91** was not seen in the starting material of other acceptors although a trace amount of oxidized product was sometimes detected.

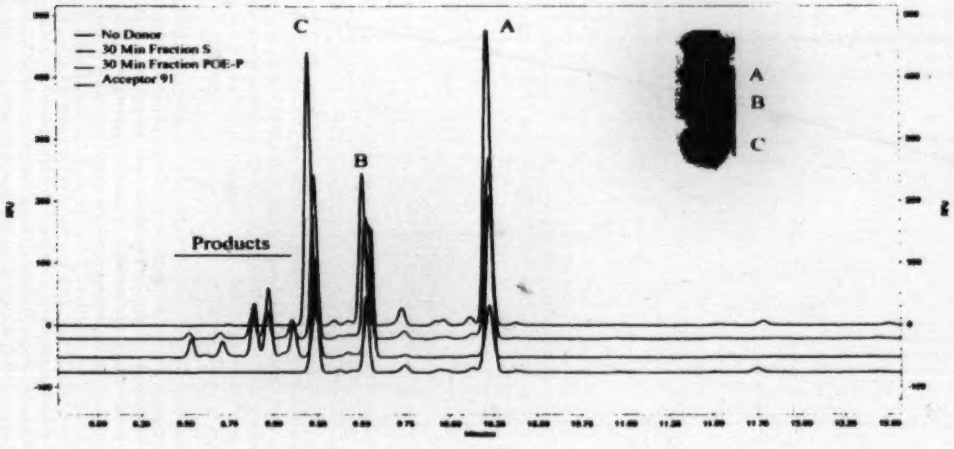


Figure 4.12 Electropherogram of the initial fungal extract screen with **91**. A, acceptor **91**; B, single oxidation of **91**; C, double oxidation of **91**. Inset: TLC of the starting material and oxidized variations.

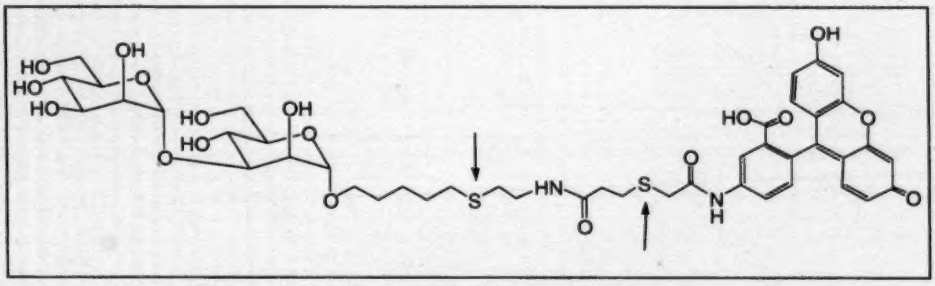


Figure 4.13 The sulphur atoms denoted by the blue arrows in the alkyl chain linking the carbohydrate to the fluorescein molecule was oxidized giving rise to a mixture of three products.

A purified and non-oxidized fraction of disaccharide **91** was assayed with various solubilization conditions of fungal extract fraction P at different pHs over time. The formation of two products was observed by TLC and CE. The use of 0.5% POE or 0.5% Triton-X with fraction P versus no detergent was monitored at pH 5.5, 6.0 and 7.0 over 24 hours. After 90 min, product 1 formation peaked (66.60%) and started to decline (Figure 4.14a). Product 2, however, was seen to increase after 90 min for all fraction P solubilized assays except 0.5% POE at pH 7.0 and attained 66.58% substrate to product conversion (Figure 4.14b). This interdependence suggested the initial construction of a trisaccharide and subsequent formation of a tetrasaccharide.

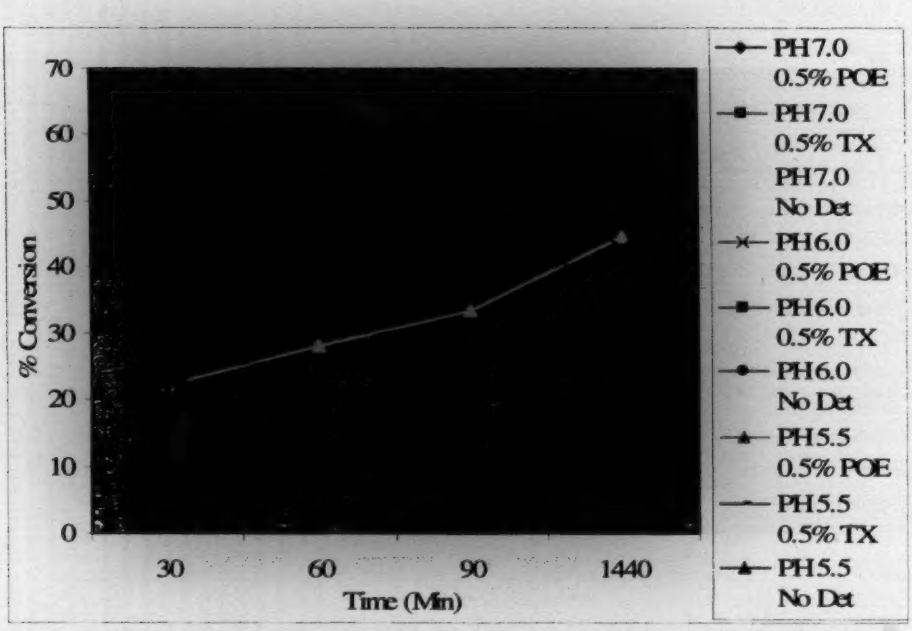


Figure 4.14a Detergent and pH optimization assay for the production of product 1 from **91**.

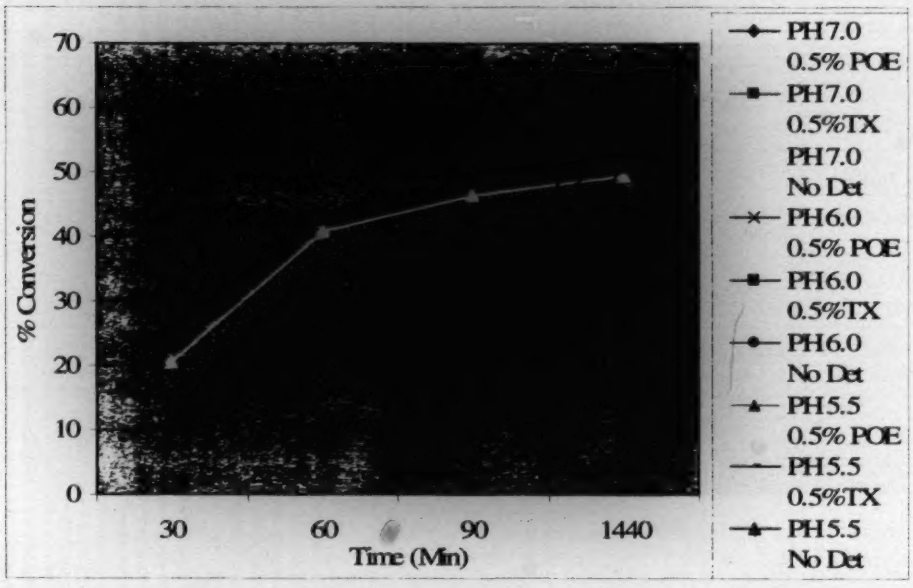


Figure 4.14b Detergent and pH optimization assay for the production of product 2 from **91**.

4.4f Large Scale Assays of 89, 90, and 91

Large scale enzymatic reactions were carried out with **89**, **90**, and **91** to determine the structure of the enzyme reaction product. Assays with α- and β-mannosidases classified the new glycosidic linkage in the product as α or β and subsequent analysis by NMR elucidated the substitution positions.

Monosaccharide acceptor **89** was incubated with α-GDP-mannose, $MnCl_2$, $MgCl_2$, DTT and fungal extract fraction P in NH_4OAc, pH 6.0, at 30 °C for eight days. Increased reaction times were again employed to maximize the conversion of substrate to product as previously discussed. Furthermore, there is precedence for a lengthened reaction time (48 h) when performing large scale enzymatic assays with these categories of substrates.[137] Only one equivalent of α-GDP-mannose was initially used; however, additional aliquots were added to the reaction over the time span shown in Table 4.7. Fraction P of the fungal extract was not solubilized as results from the optimization assay with acceptor **89** (Figure 4.9) did not demonstrate a significant difference in activity with or without the use of a detergent.

Table 4.7 Addition of α-GDP-mannose over 8 days to the large scale assay with monosaccharide **89**.

Day	0	1	2	3	4	5	6	7	8
α-GDP-mannose (Equivalents)	1	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5

The reaction was monitored by CE and showed the maximum formation of trisaccharide **93** (see p.110) as the major product (46.16%) after

eight days (Figure 4.15). Traces of di-, tetra-, and penta-saccharides as well as oxidized trisaccharide (the sulphur atom of the fluorescein linker is oxidized) were also detected (Figure 4.16). Product separation by reverse phase chromatography and subsequent MALDI analysis confirmed the various elaborations of acceptor **89**.

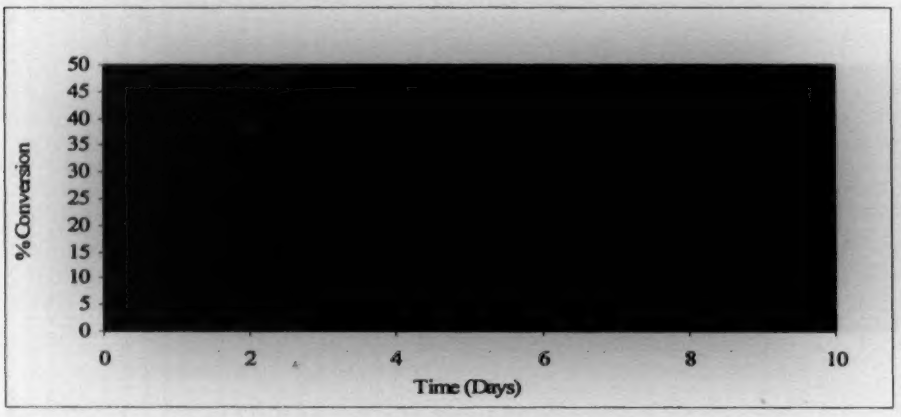


Figure 4.15 Conversion of **89** to a trisaccharide **93** over 8 days.

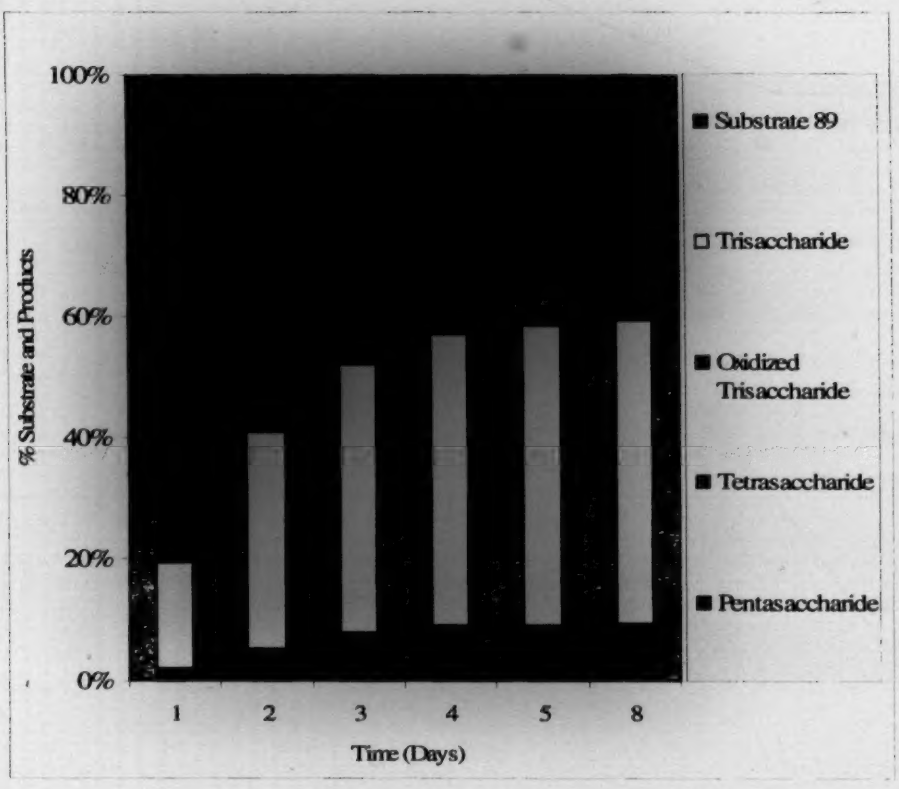


Figure 4.16 Conversion of **89** to 4 products over 8 days with fungal extract P with daily additions of α-GDP-mannose.

Disaccharide acceptor **90** was incubated with α-GDP-mannose, $MnCl_2$, $MgCl_2$, DTT and fungal extract fraction P in NH_4OAc, pH 6.0, at 30 °C for 72 hours. The reaction was supplemented with an equivalent of α-GDP-mannose at 24 and 48 hours. Fraction P of the fungal extract was again not solubilized as results from an optimization assay with acceptor **90** did not demonstrate a significant difference in activity with or without the use of a detergent. The reaction was monitored by CE and showed the maximum formation of trisaccharide **94** (16.90%) after three days (Figure 4.17). Product separation by

reverse phase chromatography and subsequent electrospray ionization high resolution mass spectroscopy (ESI HRMS) analysis confirmed the elaboration of acceptor **89** to trisaccharide **94**.

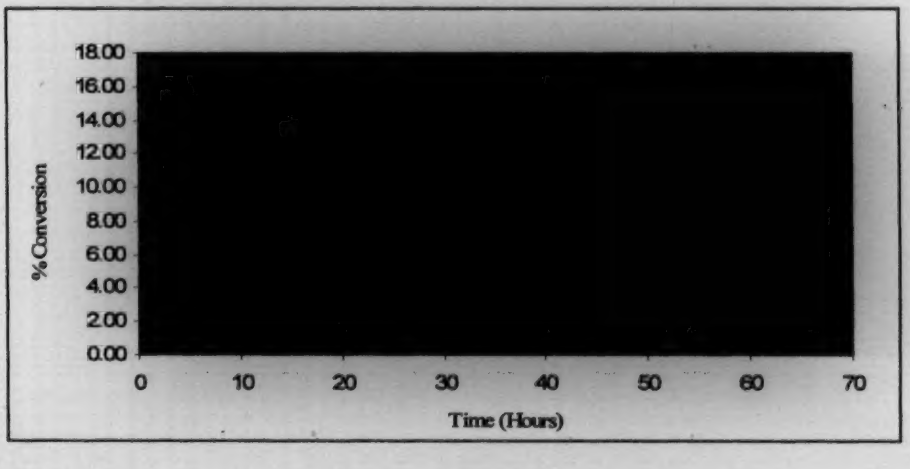


Figure 4.17 Conversion of **90** to a trisaccharide **94** over 3 days.

Disaccharide acceptor **91** was incubated with α-GDP-mannose, $MnCl_2$, $MgCl_2$, DTT and fungal extract fraction P in NH_4OAc, pH 5.5, at 30 °C for 48 hours. Two equivalents of α-GDP-mannose were used; however, they were sequentially added 18 hours apart. Fraction P of the fungal extract was again not solubilized as results from the optimization assay with acceptor **90** (Figure 4.14a and 4.14b). This assay indicated that an approximately equal mixture of the two main products could be obtained under these conditions at pH 5.5. The reaction was monitored by CE and showed the formation of three main products (39.08%, 21.04%, 25.81%) and traces of at least five other products (Figure 4.18). The appearance of a third main compound was not unexpected and was hypothesized to be the oxidized analogue of the trisaccharide product

95. Product separation by reverse phase chromatography and subsequent MALDI analysis confirmed the various elaborations of acceptor **91** trisaccharide **95** and tetrasaccharide **96**. Oligomers containing up to nine additional mannosyl units were detected.

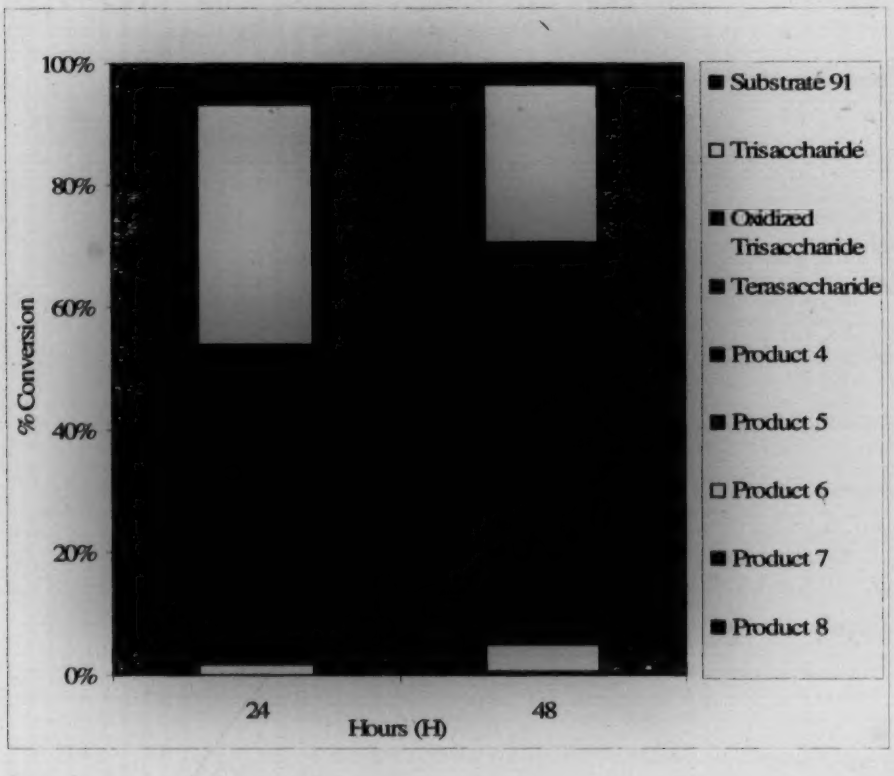


Figure 4.18 Conversion of **91** to 8 products over 2 days with α-GDP-mannose and fraction P fungal extract.

4.5 Characterization of the Products from the Large Scale Fungal Extract Assays with Acceptors 89, 90, and 91

The products from the large scale enzymatic reactions with acceptors **89, 90**, and **91** were analyzed by mannosidase digestion assays and NMR spectroscopy to determine the nature of the linkage between the new and parent residues. Jack bean α-(1→2), α-(1→3), α-(1→6)-*exo*-mannosidase and a β-mannosidase from *Cellulomonas fimi*[139] were first employed to reveal the stereochemistry of the new bonds. Successive one- and two- dimensional NMR experiments were then used to determine the substitution position on the carbohydrate rings.

4.5a Mannosidase Characterization of the Products from the Large Scale Fungal Extract Assays with Acceptors 89, 90, and 91

The large scale enzymatic assay with acceptor **89** afforded major trisaccharide product **93**, and trace amounts of the oxidized trisaccharide analogue of **93**, a tetrasaccharide, and a pentasaccharide (Figure 4.19). The mannosidase assay revealed that **93** contained two new α-linkages (Figure 4.20a). The tetra- and pentasaccharide products also revealed the sole presence of new α-linkages by mannosidase digestion (Figure 4.20b and 4.20c). Enzymatic product **93** was obtained in quantities sufficient for NMR analysis

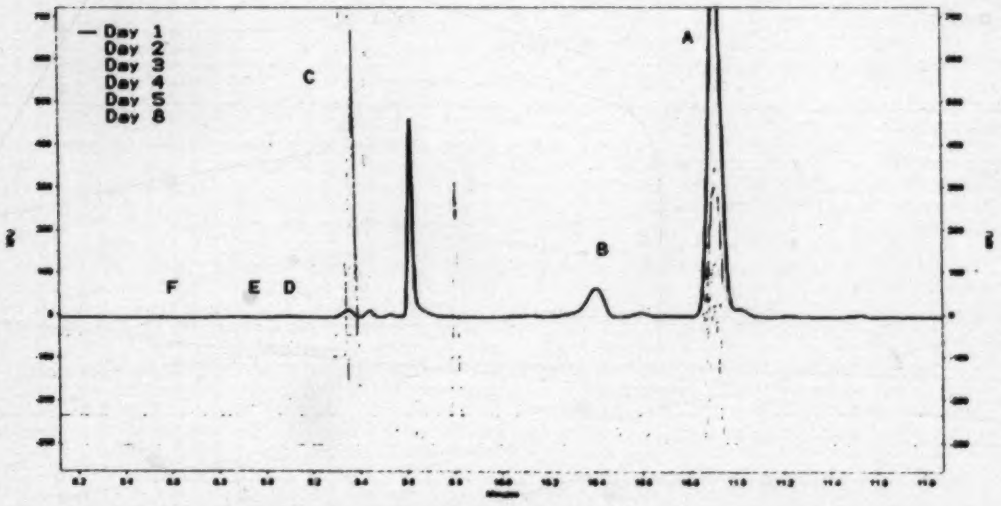
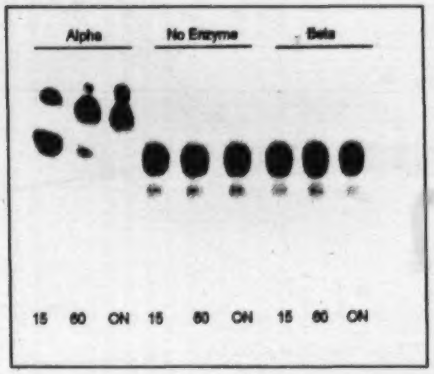
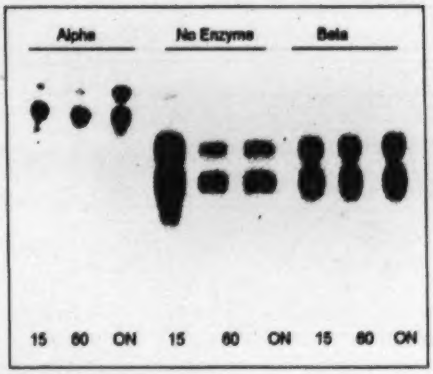


Figure 4.19 Electropherogram of the large scale enzymatic assay with acceptor **89** over eight days. A, acceptor **89**; B, oxidized analogue of **89**; C, trisaccharide **93**; D, oxidized trisaccharide; E, tetrasaccharide; F, pentasaccharide.

A



B



C

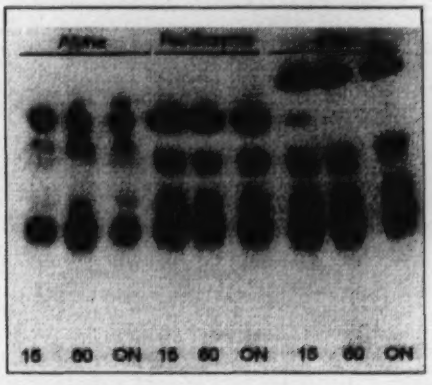


Figure 4.20 TLC of the products of the large scale enzymatic assay with acceptor **89** subjected to α- and β-mannosidase assays. (A) Trisaccharide product **93** (B) Tri- and tetrasaccharide products (C) Pentasaccharide product.

The large scale enzymatic assay with acceptor **90** afforded major trisaccharide product **94** as seen by CE (Figure 4.21). The main trisaccharide product was found to contain one new α-linkage in the mannosidase assays and was obtained in quantities sufficient for NMR analysis (Figure 4.22).

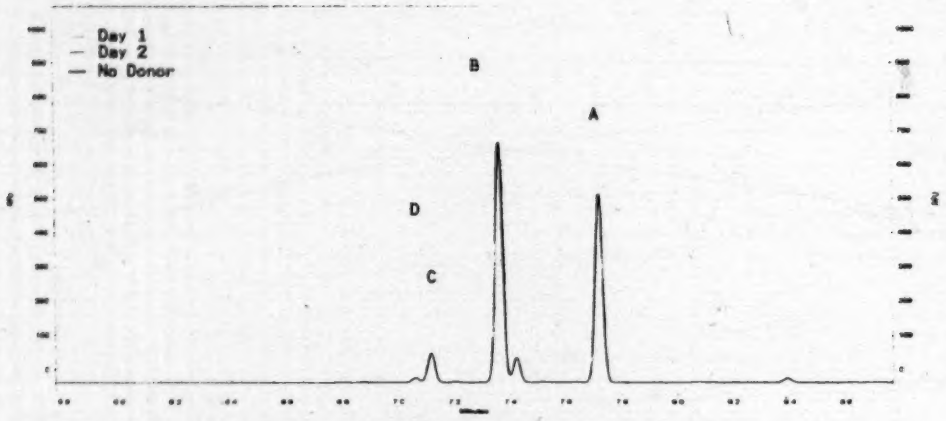


Figure 4.21 Electropherogram of the large scale enzymatic assay with acceptor **90** over two days. A, acceptor **90**; B, oxidized analogue of **90**; C, unknown; D, trisaccharide product **94**.

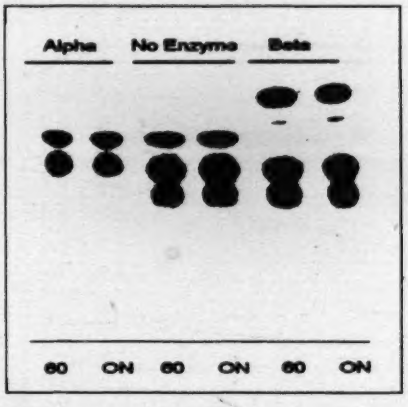


Figure 4.22 TLC of the trisaccharide product **94** of the large scale enzymatic assay with acceptor **90** subjected to α- and β-mannosidase assays.

Four products were isolated by reverse phase chromatography from the large scale reaction with acceptor **91** and were found to be tri- to

hexasaccharides (Figure 4.23). Investigation of these by the mannosidase assay revealed only the presence of α-linkages (Figure 4.24). Trisaccharide **95** and tetrasaccharide **96** were obtained in quantities sufficient for NMR analysis. Traces of hepta- to undecasaccharides were observed by MALDI but were not individually isolated.

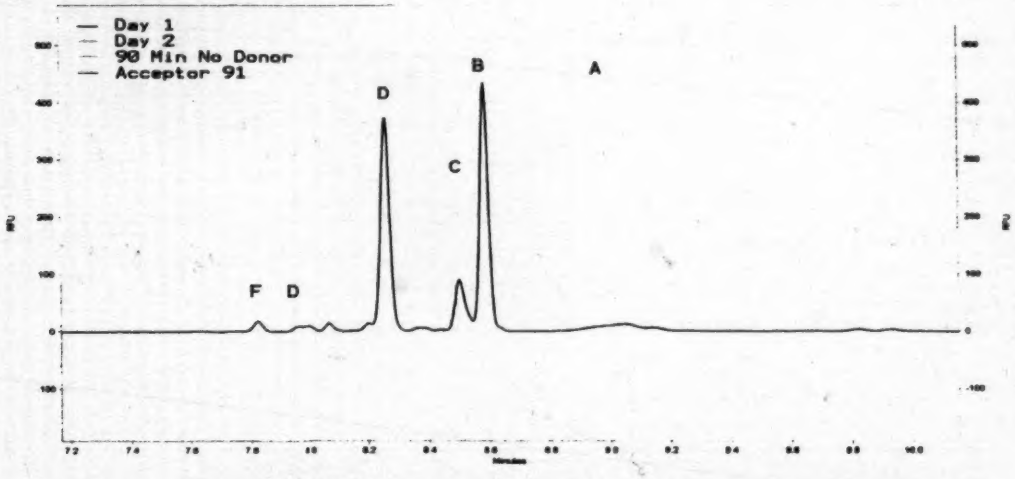


Figure 4.23 Electropherogram of the large scale enzymatic assay with acceptor **91** over two days. A, acceptor **91**; B, trisaccharide **95**; C, oxidized trisaccharide **95**; D, tetrasaccharide **96**; E, pentasaccharide; F, hexasaccharide.

A B

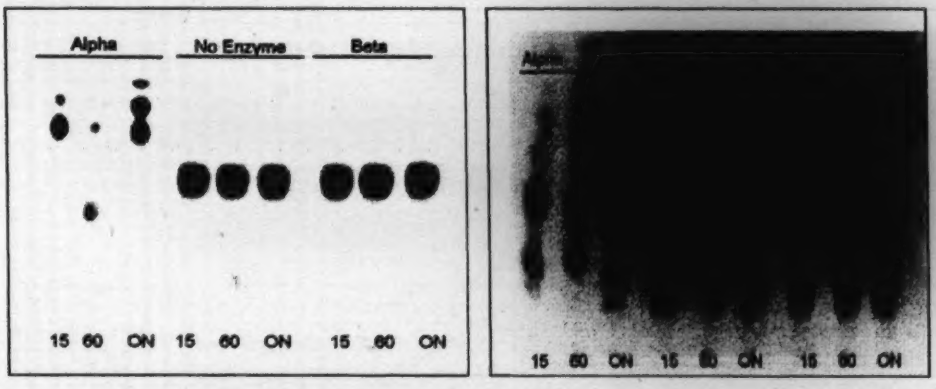


Figure 4.24 TLC of trisaccharide **95** (A) and tetrasaccharide **96** (B) products of the large scale enzymatic assay with acceptor **91** subjected to α- and β-mannosidase assays.

4.5b NMR Characterization of the Products from the Large Scale Fungal Extract Assays with Acceptors 89, 90, and 91

The structures of enzymatic products **93, 94,** and **96** were elucidated from one- and two-dimensional NMR experiments. The assignment of proton chemical shifts was achieved through GCOSY, and GTOCSY experiments. The position of the new glycosidic linkages was determined by observing the ^{13}C NMR chemical shifts in the GHSQC spectra. Data in the literature documents that carbon atoms involved in a glycosidic linkage are shifted downfield. For example, addition of an α-D-mannopyranosyl residue to the C-2 or C-3 positions of an α-D-mannopyranoside displaces the chemical shifts of the corresponding C-2 or C-3 atoms downfield by approximately 8 ppm.[140,141] Evaluation of the ^{13}C chemical shifts of acceptors **87-92** reveals the same displacement pattern (Table 4.8). The ^{13}C chemical shifts that are displaced in products **93, 94** and **96** are shown in Table 4.9.

Table 4.8 ^{13}C chemical shifts of acceptors **87-92**. ▮ Denotes that chemical shifts for the specified carbons could not be unambiguously identified. ▮ Highlights the Chemical shifts that have been displaced by substitution. R = reducing end sugar residue; NR = non-reducing end sugar residue; NNR = the sugar residue that is attached to the NR residue.

^{13}C NMR Chemical Shifts (ppm)											
	87	**88**		**89**	**90**		**91**		**92**		
	R	R	NR	R	R	NR	R	NR	R	NR	NNR
C-1	101.8	98.9	103.3	100.4	102.0	102.2	101.7	103.9	100.5	102.9	98.7
C-2	72.6	▮		71.1	▮	72.1	71.6	72.2	▮	▮	74.0
		C-2 (NR) + 2 x C-3									
C-3	75.4	71.2, 71.2, 70.8		71.4	74.6	75.3	▮	72.5	▮		77.1
C-4	68.6	67.8, 67.8		67.3	69.1	68.6	67.7	68.8	▮		
C-5	78.3	73.6	74.1	73.4	78.7, 78.5		74.9		▮		
C-6	62.9	61.9, 61.7		61.8	63.0	62.8	62.9	62.9	61.9, 61.7, 61.7		

Table 4.9 ^{13}C chemical shifts of products **93, 94** and **96.**

^{13}C NMR Chemical Shifts (ppm)		
93	C-2	75.5
	C-2'	78.1
94	C-2	78.2
	C-2'	81.0
96	C-3	79.5
	C-6	66.1
	C-2'	79.1

Enzymatic product trisaccharide **93** was previously found to contain two alpha glycosidic bonds through mannosidase digestion. GHSQC and GHMBC NMR experiments revealed that both sugar residues are linked through the O-2 positions to form the α-Man-(1→2)-α-Man-(1→2)-β-Man trisaccharide (Figure 4.25). The correlations in the GHSQC spectra were assigned with the help of GCOSY and GTOCSY experiments and comparison with the corresponding α- and β-mannopyranoside monosaccharides. The chemical shifts for C-2 and C-2' were displaced downfield from approximately 71 ppm to 75.5 ppm and 78.1 ppm respectively (Figure 4.26). This indicates that the C-2 groups of the sugar residues are glycosylated. Confirmation of the structure was achieved through a GHMBC 3-bond coupling experiment. Correlations between the anomeric proton H-1 to C-2' and between the H-1' to C-2" further supports the proposed structure for **93** (Figure 4.27).

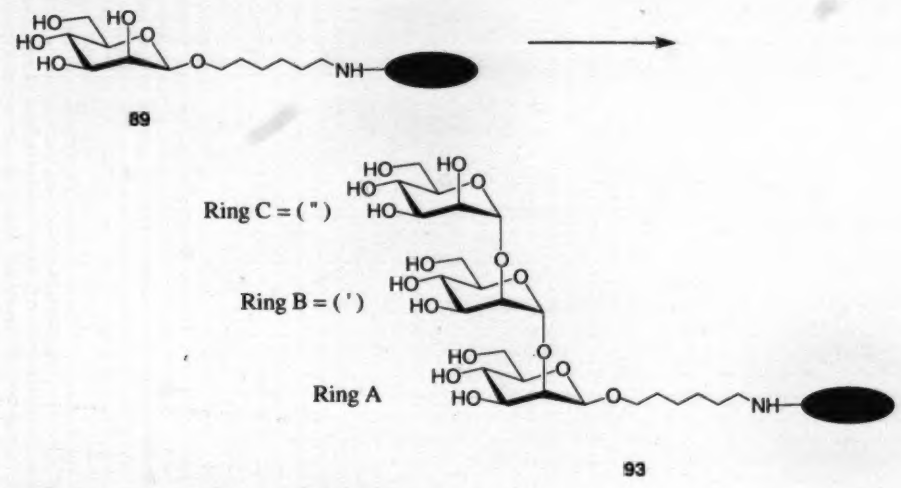


Figure 4.25 Conversion of **89** to **93.**

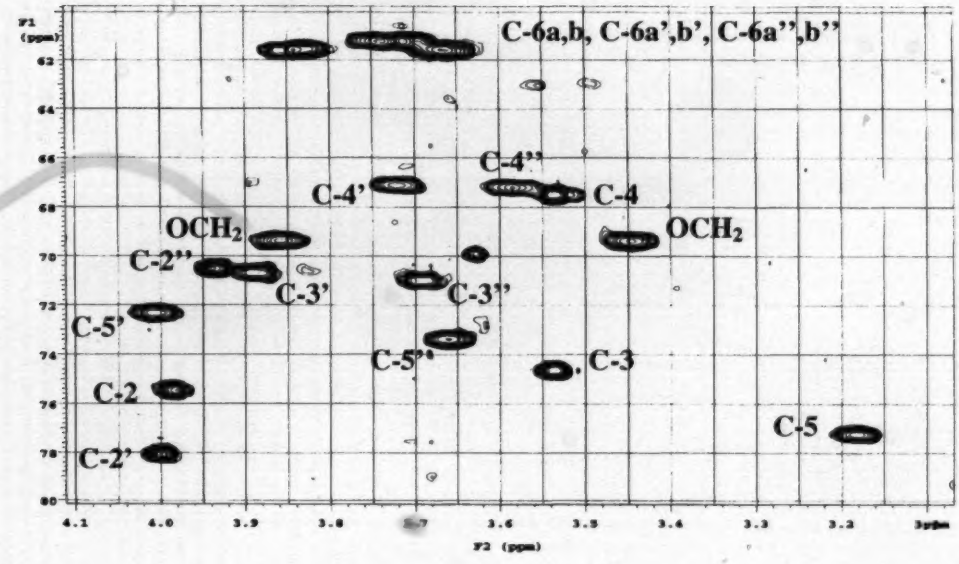


Figure 4.26 GHSQC of **93** at 600 MHz in CD_3OD. Carbon assignments highlighted in red indicate chemical shifts involved in the new glycosidic bonds.

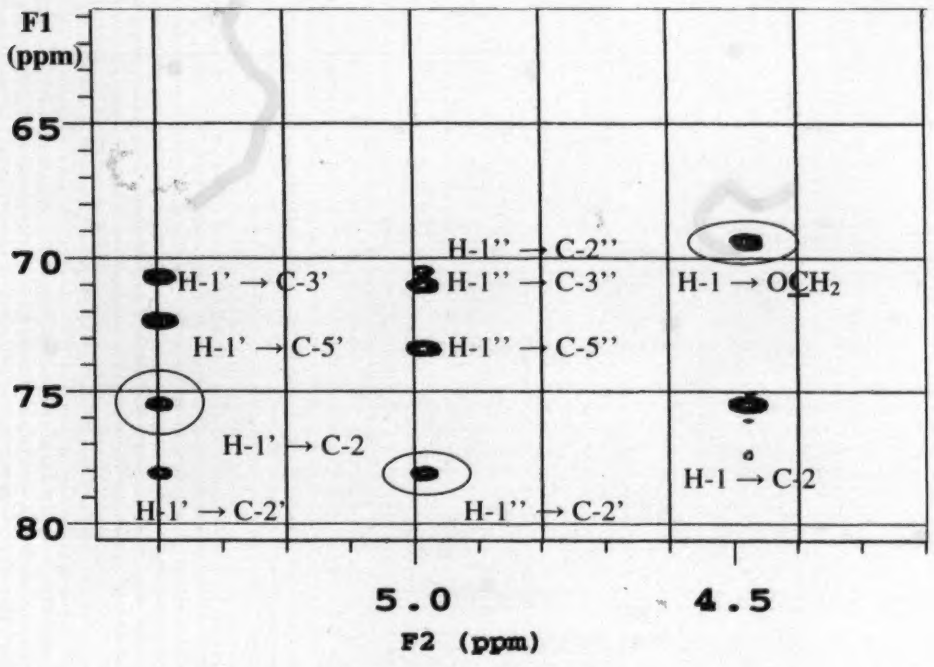


Figure 4.27 GHMBC of **93** at 600 MHz in CD_3OD. Carbon assignments highlighted in red indicate chemical shifts involved in the new glycosidic bonds.

Enzymatic product trisaccharide **94** was previously found to contain an alpha glycosidic bond by digestion with mannosidase. Unexpectedly, ^1H and GHSQC NMR experiments revealed the presence of two alpha linkages even though substrate **90**, a β-(1→2)-mannopyranoside, contained two beta linkages (Figure 4.28). The ^1H chemical shifts at 5.41 ppm, 5.00 ppm, and 4.49 ppm as well as the respective $^1J_{C\text{-}H}$ values of 175.5 Hz, 169.3 Hz, and 155.4 Hz confirm the proposed structure (Figure 4.29). The two new residues in **94** are linked through the O-2 positions to form the α-Man-(1→2)-α-Man-(1→2)-β-Man trisaccharide, the same as **93**. The correlations in the GHSQC

spectra were assigned with the help of GCOSY and GTOCSY experiments in comparison with the corresponding α- and β-mannopyranoside mono- and disaccharides. The chemical shifts for C-2 and C-2' were displaced downfield from approximately 71 ppm to 78.2 ppm and 81.0 ppm respectively (Figure 4.30). This indicates that the O-2 hydroxyl groups of the sugar residues are glycosylated. Due to the identical nature of the ^{1}H NMR experiments of **93** and **94**, further GHMBC evidence was not required. Therefore, it is hypothesized that a β-mannosidase was present in the crude fungal extract and preferentially digested the substrate before subsequent addition of new α-mannopyranosyl residues.

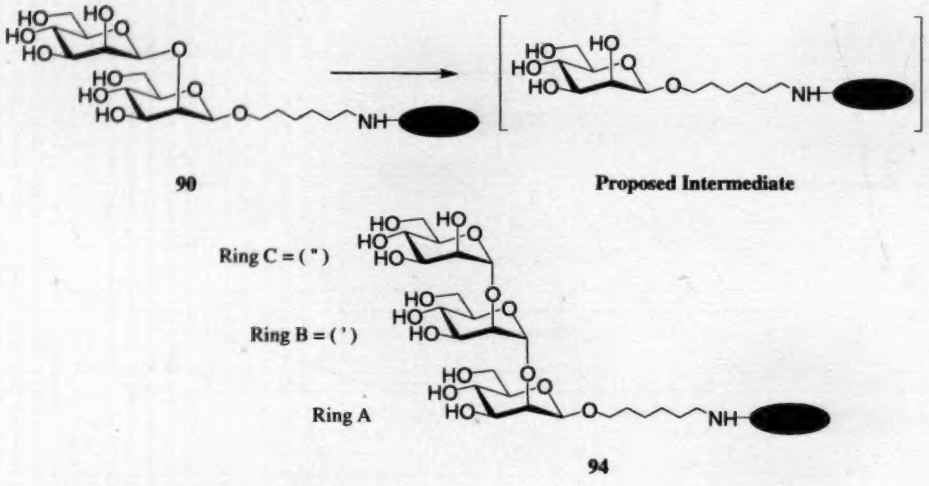


Figure 4.28 Conversion of **90** to **94**.

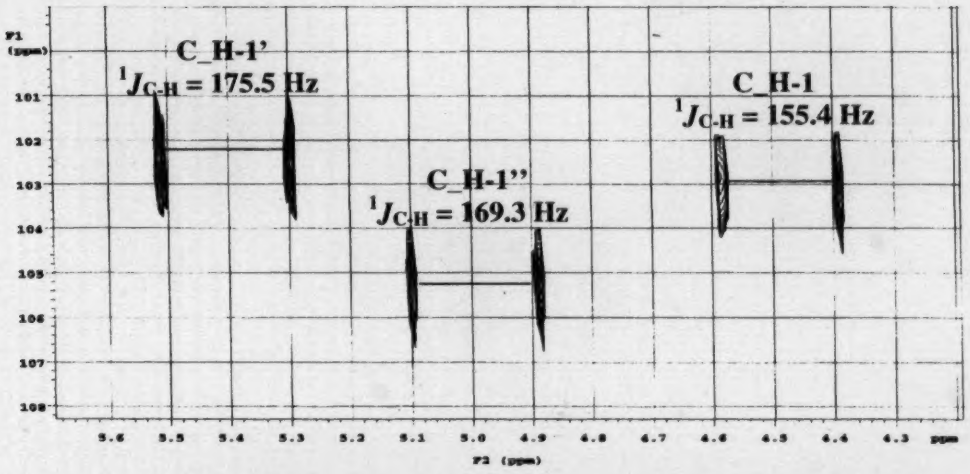


Figure 4.29 GHSQC of **94** at 800 MHz in CD_3OD. $^1J_{C-H}$ Carbon-proton assignments highlighted in blue indicate α-linkages and in red indicate a β-linkage.

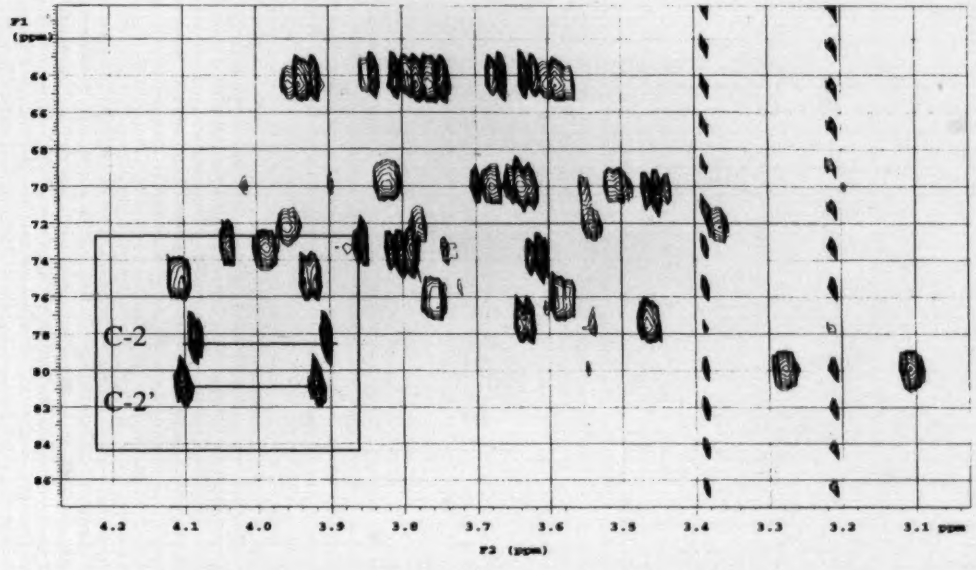


Figure 4.30 GHSQC of **94** at 800 MHz in CD_3OD. Carbon assignments highlighted in red indicate chemical shifts involved in the new glycosidic bonds.

Enzymatic products trisaccharide **95** and tetrasaccharide **96** were previously found to contain only alpha glycosidic bonds through mannosidase digestion. TLC and MALDI revealed the formation of a trisaccharide; however, ^{1}H NMR of **95** unexpectedly revealed the presence of at least two trisaccharides with different substitution patterns. Due to the difficulty of chromatographic separation, further analysis of this mixture of products was not attempted. Two structures are proposed below for **95** based on the known structure of the phosphomannan wall of *Candida albicans* (Figure 4.31, **95a** and **95b**). GHSQC and GHMBC NMR experiments with **96** revealed that the two new α-residues are linked sequentially to the reducing end through O-6 of ring system A and subsequently through O-2 of ring system B to form the branched α-Man-(1→2)-α-Man-(1→6)-[α-Man-(1→3)]-β-Man tetrasaccharide. The correlations in the GHSQC spectra were assigned with the help of GCOSY and GTOCSY experiments in comparison with the corresponding α- and β-mannopyranoside mono- and disaccharides. The chemical shifts for C-6, and C-2' were displaced downfield from 61.6 ppm and 70.6 ppm to 66.1 ppm and 79.1 ppm respectively (Figure 4.32). This indicates that O-6 and O-2' are involved in forming glycosidic bonds. Confirmation of the structure was achieved through a HMBC 3-bond coupling experiment. Correlation between the anomeric proton H-1 at 4.68 ppm to the O<u>C</u>H$_2$ of the aglycon confirms that this ring system is the reducing sugar. The presence of a correlation between H-1' and C-6 as well as between the anomeric proton H-1" to C-2' is further evidence for the structure of **93** (Figure 4.33). Furthermore, a

correlation between H-1''' to C-3 confirms the α-(1→3) linkage in the substrate is preserved. The presence of an α-(1→6) linkage is not surprising as Suzuki has determined that the α-1,6-mannopyranosyltransferase requires an α-(1→3) substrate.[131]

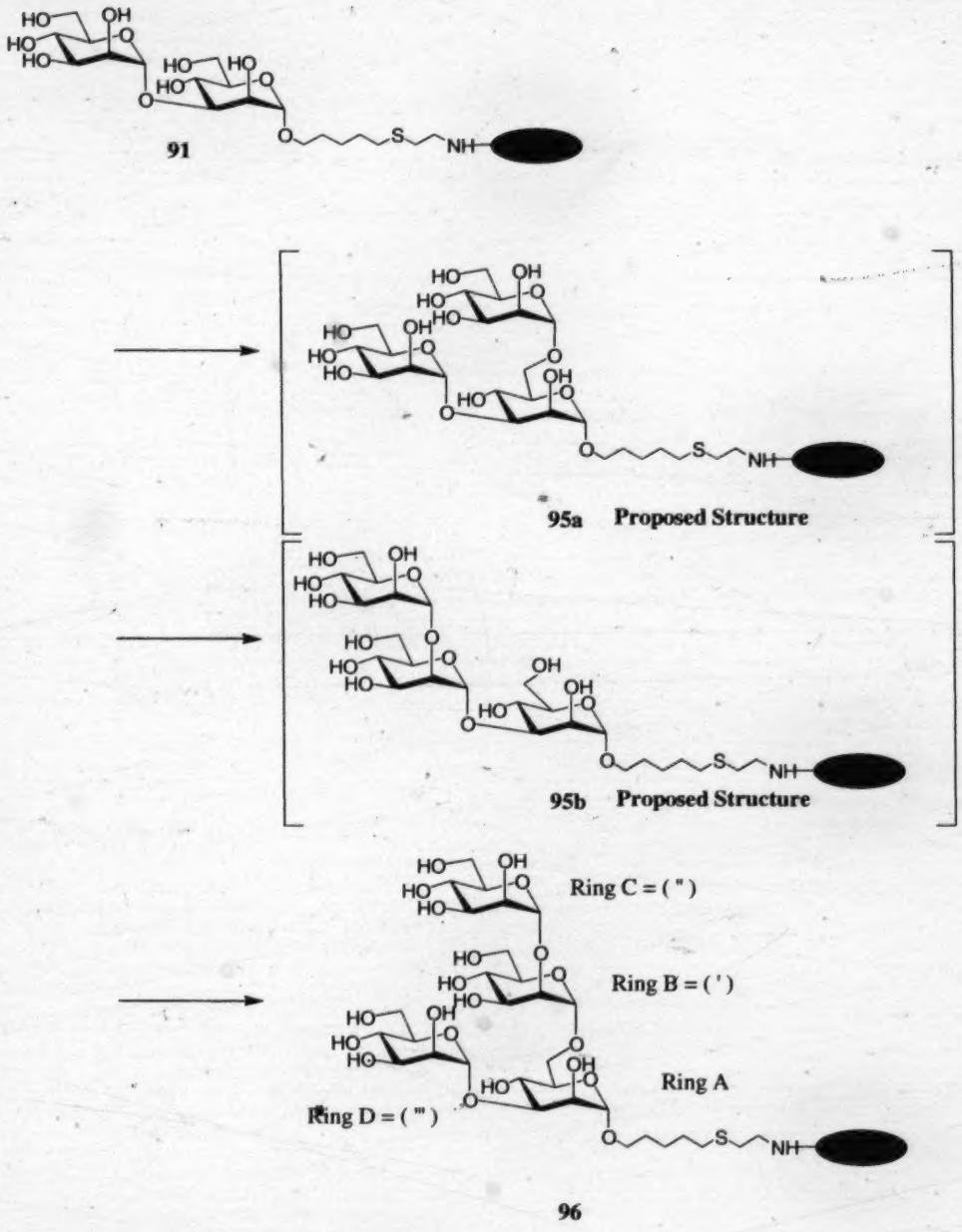


Figure 4.31 Conversion of **91** to **95** and **96**.

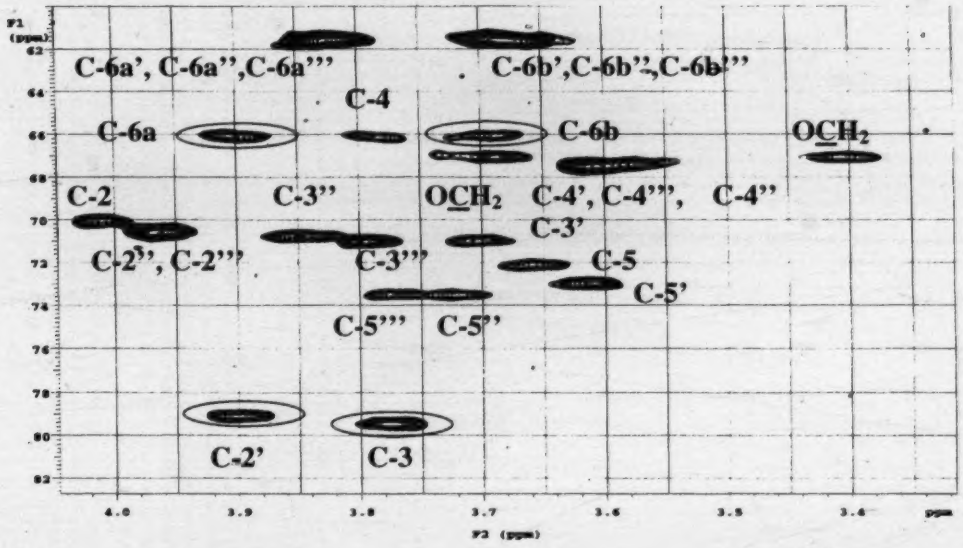


Figure 4.32 GHSQC of **96** at 600 MHz in CD_3OD. Carbon assignments highlighted in red indicate chemical shifts involved in the new glycosidic bonds and in substrate **91**.

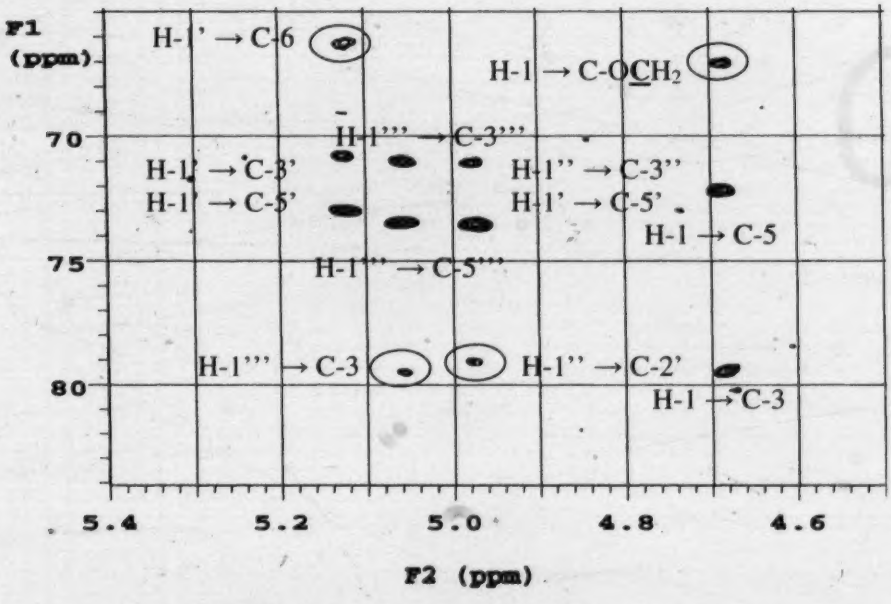


Figure 4.33 GHMBC of **96** at 600 MHz in CD_3OD. Carbon assignments highlighted in red indicate chemical shifts involved in the new glycosidic bonds and in substrate **91**.

4.6 Is the *Candida albicans* Mannopyranosyltransferase 1 Gene Product Responsible for the Products Formed in the Large Scale Assays?

The addition of α-linked mannopyranosyl residues to acceptors **89** and **91** was found to be possible using CaMNT1p; however, no significant elaboration was observed with **90**.

High conversion of acceptor **89** directly to the trisaccharide product was observed after overnight incubation with CaMNT1p, α-GDP-mannose, $MnCl_2$, and DTT in NaOAC, pH 6.0, at 30 °C (75.08%) (Figure 4.34). This was an unexpected result as this linkage sequence has not been observed in the cell wall phosphomannan of *Candida albicans* to date. Although, this enzyme has been fully characterized as an α-(1→2)mannopyranosyltransferase, the use of a β-linked substrate has not been previously reported in the literature. Although, glycosyltransferases are generally flexible towards their acceptor substrates and are very specific with regards to the linkages they form, the substitution of an α- for β- acceptor is unusual. CaMNT1p easily added the second and third α-linked mannopyranosyl residues although presence of the disaccharide was not observed by TLC or CE (Figure 4.34 inset). A fourth mannose residue was also detected on a reduced level by CE (Peak E - 5.82%). CE traces for this CaMNT1p assay show the same elution pattern observed in the large scale assay with **89**. Hence, this mannosyltransferase is most probably responsible for the same product formation and distribution.

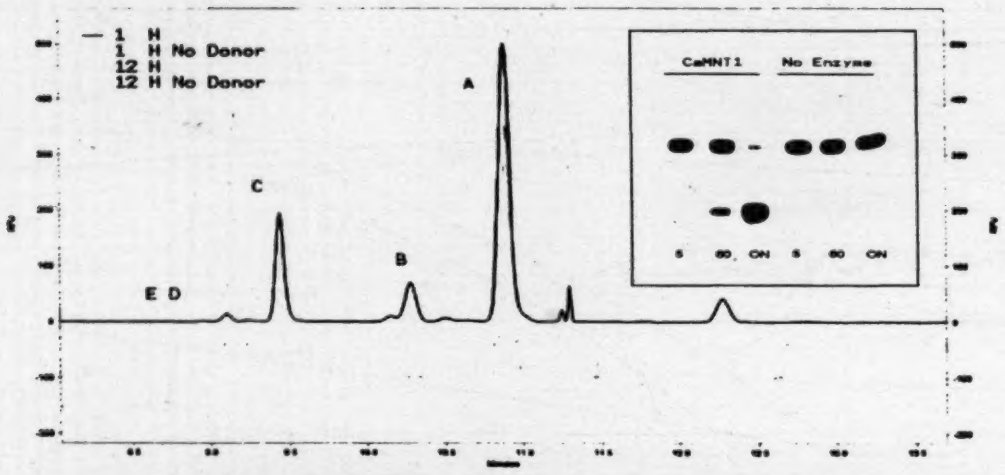


Figure 4.34 Electropherogram of the CaMNT1p assay with acceptor **89**. A, acceptor **89**; B, oxidized analogue of **89**; C, trisaccharide; D, oxidized trisaccharide; E, tetrasaccharide. Inset: TLC of the formation of a single main trisaccharide product with CaMNT1p. The disaccharide product is never observed.

A low level of transferase activity was seen with **91** in the formation of a trisaccharide product after overnight incubation with CaMnT1p, α-GDP-mannose, $MnCl_2$, and DTT in NaOAC, pH 6.0, at 30 °C (11.89%) (Figure 4.35). Previous specificity studies[133] also indicated that **91** was a much less favourable substrate than the natural α-mono- and α- (1→2)-disaccharide substrates. Therefore, a β-mannosidase must preferentially digest **91** before subsequent addition of new α-mannopyranosyl residues to form the tri- to undecasaccahrides observed with the incubation of acceptor **91** and crude fungal extract fraction P from *Candida albicans*.

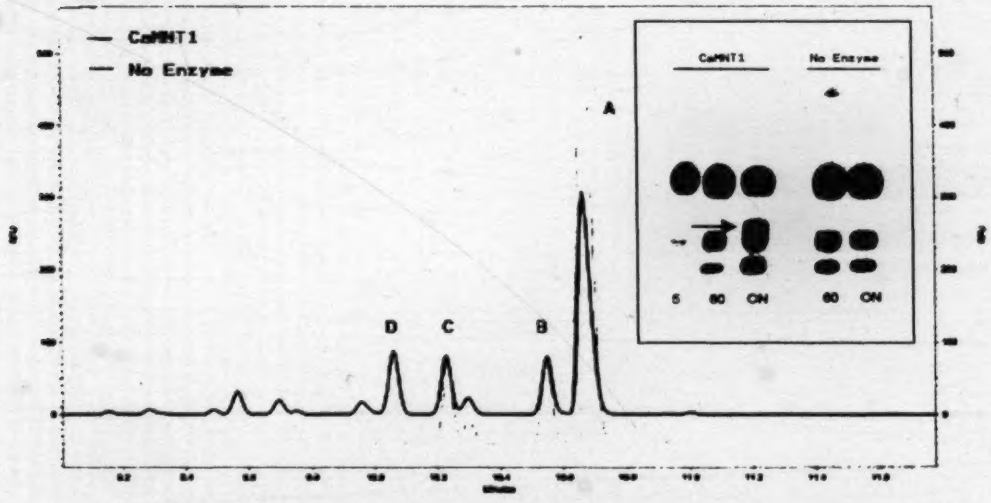


Figure 4.35 Electropherogram of the CaMNT1p assay with acceptor **91**. A, acceptor **89**; B, mono-oxidized analogue of **89**; C, di-oxidized analogue of **89;** D, trisaccharide. Inset: TLC of the the low level formation of a trisaccharide product with CaMNT1p.

The trace level of trisaccharide formed from acceptor **90** (0.35%) clearly indicates that CaMnT1p is not responsible for the transformation seen in the large scale assay (Figure 4.36). Another α-mannosyltransferase must be responsible for generating the observed reaction product.

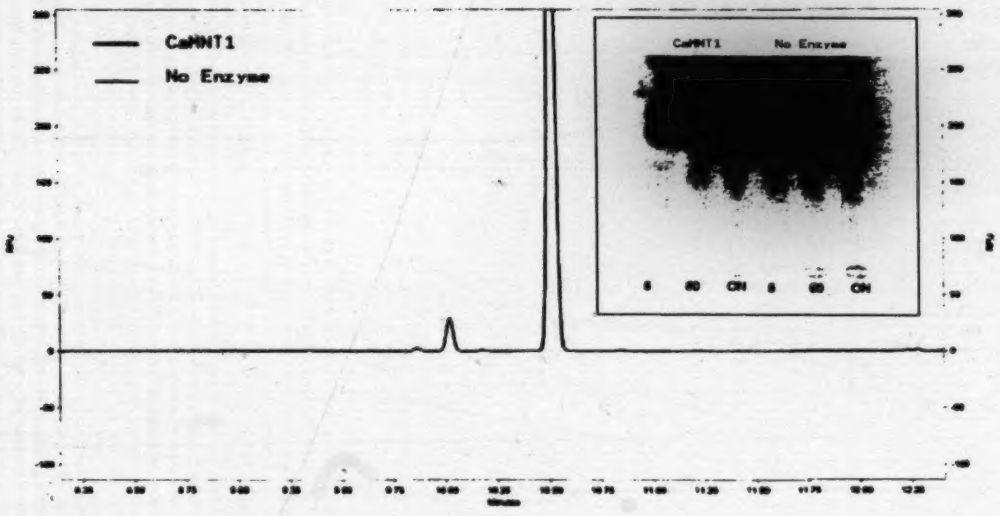


Figure 4.36 Electropherogram of the CaMNT1p assay with acceptor **90**. No products were formed as seen by CE or TLC (inset).

4.7 Summary

The fungal extract of *Candida albicans* CAI4 strain was probed with acceptors **87-92**. Mannosyltransferase activity was observed with all substrates. Subsequent optimization assays with **89**, **90**, and **91** revealed that solubilization of fungal extract fraction P did not increase the formation of product; however, an increase in product formation was observed with extended reaction times. Oxidized species of acceptor **91** made the analysis the enzymatic products difficult. Use of the 6-aminohexyl linker with **89** and **90** fortuitously did not lead to the same problem. Large enzymatic assays were done with acceptors **89**, **90**, and **91** to generate analytical quantities of **93**, **94** and **96** to more specifically probe the nature of the linkage between the new

mannose unit and the parent chain. Through mannosidase digestion and NMR analysis, the structure of each product was elucidated. Identical trisaccharides **93** and **94** are not found naturally in the phosphomannan of *Candida albicans*. When presented with a β-mannopyranosyl O-2, another β-mannopyranosyl residue should have been added to form antigenic factor 5. Tetrasaccharide **96** is related to a structure found in the phosphomannan; however, the fourth mannopyranosyl residue should have been added to O-2''' (ring system D) instead of O-2'' (ring system C). The formation of unnatural structures *in vitro* suggests that the mannopyranosyltransferases detected in these assays have become less specific for their substrates or have become deregulated in their altered environment. From the CE results of the assays with CaMNT1p, this enzyme is most probably responsible for the formation of **93** and **94** and perhaps plays a role in the construction of **96**.

Chapter 5

Conclusions and Future Directions about the *Candida albicans* Mannopyranans and the Preliminary Steps Towards the Identification of β-Mannopyranosyltransferases

The synthesis of several mannopyranan acceptors was achieved through novel and literature protocols. Lewis acid promoted orthoester rearrangement and ultrasound promoted axial nucleophilic displacement were employed to synthesize β-mannopyranosides. Application of these optimizations afforded β-(1→2)-mannopyranosides with greater ease and in higher yield. This thesis reports the first use of the 6-aminohexyl linker with α- and β-mannopyranosides. Fortuitously, this linker was more easily installed and was much better suited to enzymatic reaction conditions due to the absence of a sulphur atom in comparison to the 2-aminoethanethiol linker, used in literature.[117] Partial oxidation of the sulphur atoms led to complicated analysis as the starting material and associated products were distributed into a range of compounds; this was especially the case with the α-(1→3)-mannopyranoside **91**.

Screening of the fungal extract of *Candida albicans* with the chemically synthesized acceptors revealed α-mannopyranosyltransferase and β-mannosidase activity. The formation of α-(1→2)- and α-(1→6)-

mannopyranosidic linkages indicates that at least two mannopyranosyltransferases are present in fraction P of the crude fungal extract. Furthermore, the structure of the enzymatic product with the β-(1→2)-mannopyranoside **90** acceptor indicates that a β-mannosidase hydrolysed the non-reducing sugar of the acceptor before subsequent elaboration. The products formed in the large scale assays are not found naturally in the phosphomannan. Therefore, it is suggested that these enzymes are relaxed in their substrate specificity but are still specific for the linkage they form. The absence of β-mannopyranosyltransferase activity suggests that either different reaction conditions are required, the synthesized acceptors are not substrates, or that the enzymes have been denatured during the extraction process.

Suzuki and coworkers have been successful in observing β-mannopyranosyltransferase activity with *C. albicans* serotype A strain, J-1012, and extracted natural substrates.[132] In the future, the use of this strain of *Candida albicans* may increase the chances of detecting β-mannopyranosyltransferase activity and would confirm the suitability of the synthesised acceptors. The literature and results from this thesis suggest that longer oligomannopyranosides or substrates containing a phosphate group are required for β-mannopyranosyltransferase activity. Furthermore, growth of *C. albicans* at pH 4.5 has led to the formation of β-(1→2)-mannopyranosides with much higher degrees of polymerisation than observed in nature.[142] It is tempting to speculate that this increase in β-mannopyranosyltransferase

activity under these growth conditions could ease the detection of β-mannopyranosyltransferases *in vitro*.

CHAPTER 6

Experimental Conditions

6.1 General Synthetic Conditions and NMR Numbering Scheme

6.1a Chemical Synthesis

Commercial reagents were used as supplied from Sigma-Aldrich, Fisher Scientific, Fluka, and Molecular Probes. Reactions were carried out at the temperatures stated. Solvents were concentrated *in vacuo* between 20 and 40 °C bath temperature. Glycosylation reactions used 4Å molecular sieves and stir bars that were flame dried and cooled under high vacuum. Donors and acceptors were mixed and subsequently dried together under reduced pressure. The anhydrous conditions also employed a positive pressure of argon and solvents that were dried over activated alumina using a solvent purification system from Innovative Technology (SPS-400, Newburyport, USA). Photoaddition of 2-aminoethanethiol hydrochloride to allyl glycosides was carried out using cylindrical quartz vessels and a spectroline model ENF-2400 UV lamp. Ultrasound promoted C-2 inversion was achieved using cylindrical glass vessels and a Bransonic (B-32) ultrsonic cleaning bath. Conjugation of the fluorescein moiety to the free amines was carried out in 1.5mL micro centrifuge tubes. Analytical thin layer chromatography (TLC) was performed on silica gel 60-F_{254} (Merck). Compounds were visualized under UV light, and/or by treatment with either acidic cerium ammonium molybdate or 5% ethanolic sulfuric acid, followed by charring with heating.

6.1b Chromatography

Medium pressure column chromatography used silica gel (SiliCycle, 230-400 Mesh, 60Å) and ACS grade solvents. Reverse phase C_{18} silica gel cartridges (C_{18} SepPak) were obtained from Waters Corp. (Milford, USA). High performance liquid chromatography (HPLC) was conducted with a Waters Delta 600 system using a tunable UV absorbance detector. Separations were achieved with Beckman C_{18}-silica semi-preparative or Hamilton PRP-1 reverse phase semi-preparative columns. Combinations of water and methanol or water and acetonitrile spiked with 0.1% triethylamine or acetic acid (flow rate 1.5-2.0 mL min^{-1}) were used as eluents.

6.1c Analytical

^{1}H and ^{13}C NMR spectra were recorded on Varian INOVA 500 and 600 MHz spectrometers. First order ^{1}H NMR chemical shifts are reported in δ (ppm) units and are referenced to internal standards of the residual protonated solvent peaks; δ_H 7.24 ppm for solutions in $CDCl_3$, δ_H 3.30 ppm for solutions in CD_3OD, and 0.1% external acetone (δ_H 2.225 ppm) for solutions in D_2O. Assignments were made with the aid of GCOSY, GHMQC, GHMBC, and GTOCSY experiments. ^{13}C NMR spectra were recorded at 150 MHz and are referenced to internal $CDCl_3$ (δ_C 77.0 ppm), CD_3OD (δ_C 49.0 ppm), or to external acetone (δ_C 31.07 ppm). ^{13}C chemical shifts are reported to the second decimal place, although it is expected that the reproducibility of chemical shifts will only be accurate to one tenth of a ppm. The reported data

often include resonances separated by less than 0.1 ppm. Labelling schemes for the atoms of the allyl orthoester and the anomeric alkyl chains and fluorescent moiety are indicated in Figures 6.1a, b, c, d and e.

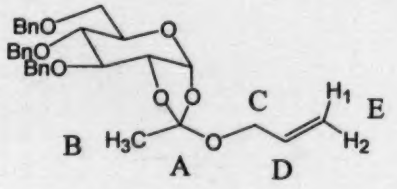


Figure 6.1a Labelling of the orthoester **58**.

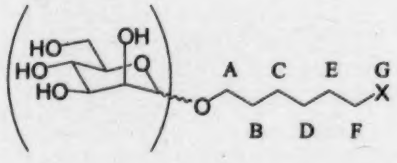


Figure 6.1b Labelling of the aglycon where X = N_3 or NH_2.

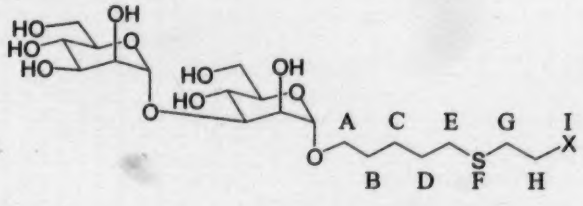


Figure 6.1c Labelling of the aglycon where X = N_3 or NH_2.

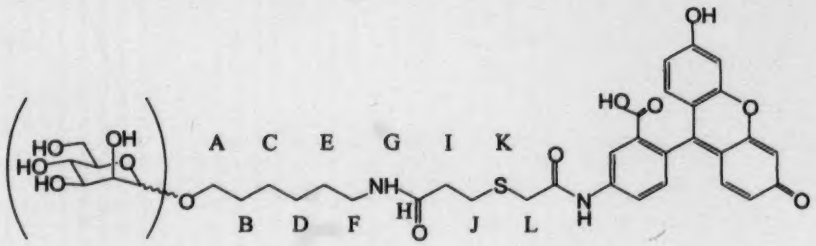


Figure 6.1d Labelling of the aglycon for **87- 90**, and **92**.

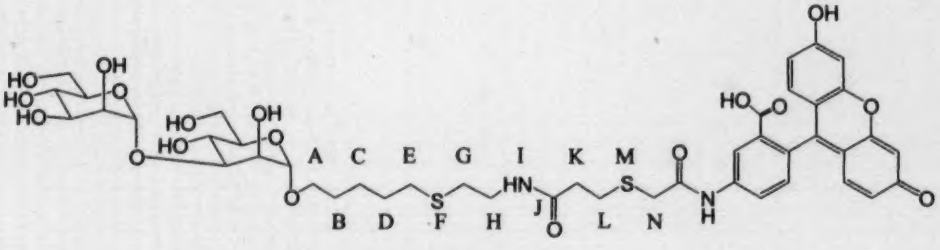
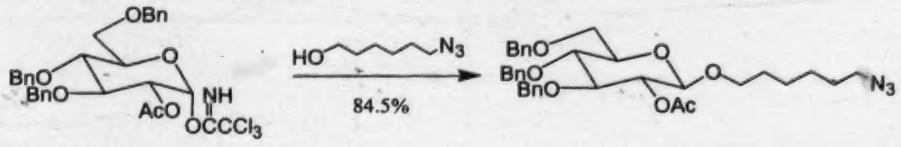


Figure 6.1e Labelling of the aglycon for **91**.

Optical rotations were measured with a Perkin Elmer 241 polarimeter at 22 ± 2 °C. Electrospray ionization mass spectra were acquired at the mass spectrometry laboratory of the Department of Chemistry. Spectra were recorded on a Micromass Zabspec TOF mass spectrometer. For high resolution mass determination, spectra were obtained by voltage scan over a narrow range at a resolution of approximately 10^4.

6.2 Synthetic Chemistry

6′-Azidohexyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranoside (**57a**).

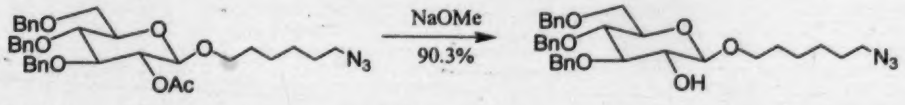


62 **57a**

Trichloroacetimidate glucopyranosyl donor **62** (2.014g, 3.16mmol) and 6-azido-1-hexanol (0.992g, 6.93mmol) were dried together under vacuum for 1 h before dissolution in dry CH_3CN (10.5mL). 4Å Molecular sieves (0.9927g) and a stir bar were previously flame dried and left under vacuum for 2 h before addition to the reaction solution. The mixture was stirred for 1 h under argon at room temperature before being cooled to 0 °C and stirred for 10 more min. TMSOTf (100μL, 0.5525mmol) was added dropwise from a syringe. After being stirred for an additional 30 min, the reaction mixture was neutralized with Et_3N, concentrated to dryness, and purified by column chromatography in 20:1 toluene-ethyl acetate. Glucopyranoside **57a** was obtained as a syrup (1.651g, 85%); $[\alpha]_D^{25}$ +6.6 (*c* 3.31, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H, 7.38-7.15 (m, 15H, aromatic), 5.0 (high order t, 1H, H-2), 4.80 (d, 2H, ^2J = 11.1 Hz, 2 × OCH_2Ph), 4.68 (d, 1H, ^2J = 11.4 Hz, OCH_2Ph), 4.64 (d, 1H, ^2J = 12.1 Hz, OCH_2Ph), 4.57 (d, 2H, ^2J = 12.2 Hz, 2 × OCH_2Ph), 4.36 (d, 1H, J = 8.0 Hz, H-1), 3.88 (ddd, 1H, J = 6.2, 6.2, 9.6 Hz, H-A1), 3.76 (dd, 1H, J = 2.0, 10.9 Hz, H-6a), 3.72 (dd, 1H, J = 4.9, 10.9 Hz, H-6b), 3.70-3.64 (m, 2H, H-3, H-4), 3.50 (m, 1H, H-5), 3.46 (ddd, 1H, J = 6.6, 6.6, 9.6 Hz,

H-A2), 3.26 (t, 2H, J = 6.9 Hz, H-F1, H-F2), 1.97 (s, 3H, OAc); 1.66-1.51 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.43-1.30 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 169.40 (C=O), 138.22, 138.16, 137.91 ($\underline{C}_6H_5CH_2O$), 28.43, 128.37, 128.03, 127.84, 127.73, 127.62 ($\underline{C}_6H_5CH_2O$), 101.0 (C-1, $^1J_{C\text{-}H}$ = 158.7 Hz), 83.01 (C-3), 78.09 (C-4), 75.22 (C-5), 75.05, 75.01, 73.52 ($O\underline{C}H_2Ph$), 73.21 (C-2), 69.35 (C-A), 68.86 (C-6), 51.34 (C-F), 29.36, 28.79 (C-B and C-E), 26.44, 25.50 (C-C and C-D), 20.91 (Ac); ESI HRMS (m/z): Calcd for $C_{35}H_{43}N_3O_7Na$ 973.4497, found 973.4498.

6′-Azidohexyl 3,4,6-tri-*O*-benzyl-β-D-glucopyranoside (**57b**).

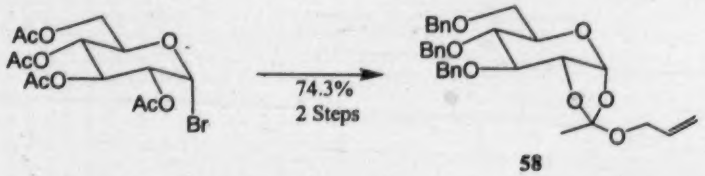


57a **57b**

57a (1.651g, 2.673mmol) in dry MeOH (50mL) was treated with NaOMe (3.5 M, 1.2mL) and heated at reflux for 1 h. The reaction solution was neutralized with Amberlite IR 120 resin beads, filtered, and concentrated *in vacuo*. Flash silica gel chromatography of the residue with 4:1 toluene-ethyl acetate afforded the selectively protected glucopyranoside **57b** (1.389g, 74% 2 steps); $[\alpha]_D^{25}$ -4.0 (*c* 1.04, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H, 7.41-7.16 (m, 15H, aromatic), 4.94 (d, 1H, ^2J = 11.3 Hz, OCH_2Ph), 4.85 (d, 1H, ^2J = 11.4 Hz, OCH_2Ph), 4.84 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.63 (d, 1H, ^2J = 12.3 Hz, OCH_2Ph), 4.56 (d, 1H, ^2J = 12.3 Hz, OCH_2Ph), 4.55 (d, 2H, ^2J = 10.8 Hz, OCH_2Ph), 4.26 (d, 1H, J = 7.5 Hz, H-1), 3.93 (ddd, 1H, J = 6.2, 6.6 , 9.6 Hz, H-A1), 3.76 (d, 1H, J = 2.0, 10.8 Hz, H-6a), 3.70 (d, 1H, J = 4.8, 10.8 Hz, H-

6b), 3.64-3.51 (m, 4H, H-2, H-3, H-4, H-A2), 3.50 (m, 1H, H-5), 3.27 (t, 2H, J = 6.9 Hz, H-F1, H-F2), 2.32 (s, 1H, OH), 1.74-1.54 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.46-1.36 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 138.66, 138.16, 138.09 ($\underline{C}_6H_5CH_2O$), 128.46, 128.39, 128.35, 127.96, 127.93, 127.77, 127.71, 127.62 ($\underline{C}_6H_5CH_2O$), 102.74 (C-1, $^1J_{C\text{-}H}$ = 158.0 Hz), 84.56 (C-3), 77.65 (H-4), 75.21 (C-5), 75.09, 75.00 ($O\underline{C}H_2Ph$), 74.73 (C-2), 73.51 ($O\underline{C}H_2Ph$), 69.81 (C-A), 68.98 (C-6), 51.38 (C-F), 29.47, 28.76 (C-B and C-E), 26.49, 25.61 (C-C and C-D); ESI HRMS (m/z): Calcd for $C_{33}H_{41}N_3O_6Na$ 598.289, found 598.289.

3,4,6-tri-*O*-benzyl-1,2-*O*-(*exo*-allyloxy)-ethylidene-α-D-glucopyranoside (**58**)

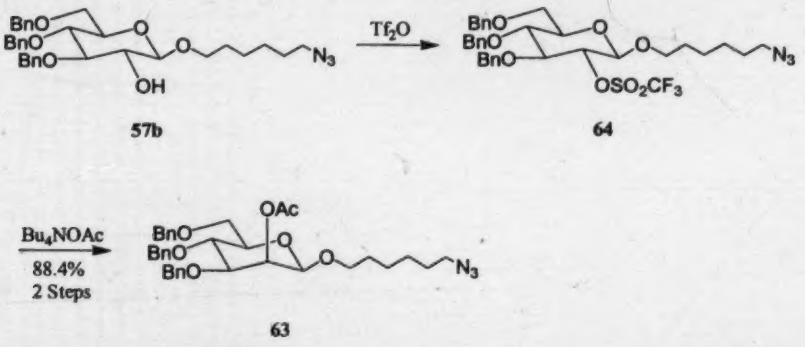


58

2,3,4,6,-tetra-*O*-acetyl glucopyranosyl bromide (14.01g, 34.07mmol) was dissolved in freshly distilled CH_2Cl_2 (90mL). 2,6-Lutidine (6.4mL, 55mmol), allyl alcohol (9.9mL, 146mmol), and TBABr (1.29g, 4.00mmol) were added to the solution. The reaction mixture was heated at reflux and monitored by TLC in 10:0.3 chloroform-acetone with 2 drops of Et_3N. After 48 h, the reaction solution was diluted with EtOAc and washed with saturated $NaHCO_3$. The resulting organic extract was dried over Na_2SO_4, filtered, concentrated under vacuum and purified by chromatography using 2:1 hexanes-ethyl acetate with 2% Et_3N as eluant. Part of the purified residue (0.328g,

0.844mmol) was dissolved in THF (5mL). Benzyl bromide (0.42mL, 3.5mmol) and potassium hydroxide (0.712g, 12.7mmo) were added and the resulting solution was heated at reflux. After 3 h, the reaction mixture was diluted with EtOAc, successively washed with water and saturated brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and purified by chromatography using 6:1 hexanes-ethyl acetate as eluant. Orthoester **58** was obtained as a syrup (0.404g, 90%); $[\alpha]_D^{25}$ +71.1 (*c* 1.75, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ_H, 7.40-7.18 (m, 15H, aromatic), 5.91 (m, 1H, H-D), 5.78 (d, 1H, J = 5.2 Hz, H-1), 5.29 (m, 1H, H-C2), 5.16 (m, 1H, H-C1), 4.71 (d, 1H, ^2J = 11.9 Hz, OCH_2Ph), 4.61 (d, 2H, ^2J = 11.8 Hz, OCH_2Ph), 4.59 (d, 1H, ^2J = 12.2 Hz, OCH_2Ph), 4.52 (d, 1H, ^2J = 12.2 Hz, OCH_2Ph), 4.44 (dd, 1H, J = 3.6, 4.8 Hz, H-2), 4.40 (d, 1H, ^2J = 11.5 Hz, OCH_2Ph), 4.09-4.00 (m, 2H, H-C1, H-C2), 3.89 (dd, 1H, J = 4.5, 4.5 Hz, H-3), 3.80 (ddd, 1H, J = 2.3, 3.8, 9.5 Hz, H-5), 3.72 (dd, 1H, J = 4.4, 9.4 Hz, H-4), 3.68 (dd, 1H, J = 2.3, 10.8 Hz, H-6a), 3.65 (dd, 1H, J = 4.0, 10.8 Hz, H-6b), 1.69 (s, 3H, H-Ba, H-Bb, H-Bc); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 138.11, 137.94, 137.72 ($\underline{C}_6H_5CH_2O$), 134.40 (C-D), 128.45, 128.40, 128.34, 128.33, 128.30, 128.07, 128.01, 127.90, 127.84, 127, 77, 127.59 ($\underline{C}_6H_5CH_2O$), 121.10 (C-A), 116.54 (C-E), 97.84 (C-1), 78.73 (C-3), 75.81 (C-2), 74.93 (C-4), 73.42, 72.94, 71.92 ($O\underline{C}H_2Ph$), 70.55 (C-5), 69.19 (C-6), 64.35 (C-C), 21.97 (C-B); ESI HRMS (*m/z*): Calcd for $C_{32}H_{36}O_7Na$ 555.2353, found 555.2350.

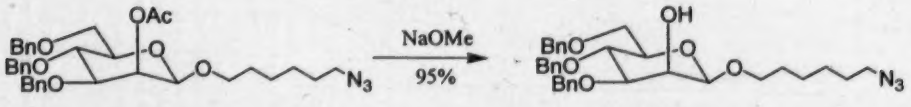
6′-Azidohexyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (**63**).



A solution of **57b** (0.480g, 0.833mmol), in dry CH_2Cl_2 (12mL), and dry pyridine (10mL) was stirred at –40 °C for 10 min. Trifluoroacetic anhydride (0.34mL, 2.444mmol) was added dropwise and the reaction mixture slowly warmed to –7 °C over 2 h and further stirred for 2 h at this temperature. The solution was finally warmed to 10 °C and diluted with CH_2Cl_2 before successive washing with aqueous HCl (0.5 M) and brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give crude **64**. The residue was further dried under high vacuum for 1h. Intermediate **64** was dissolved in dry toluene (10mL) and Bu_4NOAc (0.247g, 0.820mmol) was added. The reaction vessel was flushed with argon and the reaction mixture sonicated overnight in an ultrasound cleaning bath. The solution was concentrated under reduced pressure and purified by chromatography using first pure hexanes and then a mixture of 12:1 toluene-ethyl acetate as eluents. The mannopyranoside **63** was obtained as a syrup (0.455g, 88%); $[\alpha]_D^{25}$ +12.9 (*c* 0.86, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ_H, 7.37-7.15 (m, 15H, aromatic), 5.61 (d, 1H, J = 3.3 Hz, H-2), 4.87 (d, 1H, ^2J = 10.7 Hz, OCH_2Ph),

4.76 (d, 1H, ^2J = 11.1 Hz, OCH_2Ph), 4.64 (d, 1H, ^{2}= 12.1 Hz, OCH_2Ph), 4.56 (d, 1H, ^2J = 12.2 Hz, OCH_2Ph), 4.51 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.50 (d, 1H, ^2J = 11.2 Hz, OCH_2Ph), 4.49 (d, 1H, $J \leq 1$ Hz, H-1), 3.98 (ddd, 1H, J = 6.5, 6.5, 9.3 Hz, H-A1), 3.79 (dd, 1H, J = 1.9, 10.8 Hz, H-4), 3.77-3.71 (m, 2H, H-6a, H-6b), 3.66 (dd, 1H, J = 3.2, 9.3, H-3), 3.52-3.45 (m, 2H, H-5, H-A2), 3.25 (t, 1H, J = 7.0 Hz, H-F1, H-F2), 2.20 (s, 3H, OAc), 1.66-1.49 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.44-1.33 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 170.63 (C=O), 138.29, 138.19, 137.59 ($\underline{C}_6H_5CH_2O$), 128.43, 128.33, 128.31, 128.16, 127.95, 127.82, 127.70, 127.57 ($\underline{C}_6H_5CH_2O$), 98.95 (C-1, $^1J_{C\text{-}H}$ = 154.7 Hz), 80.42 (C-3), 75.60 (C-4), 75.16 ($O\underline{C}H_2Ph$), 74.45 (C-5), 73.21, 71.47 ($O\underline{C}H_2Ph$), 69.73 (C-A), 69.39 (C-6), 68.06 (C-2), 51.37 (C-F), 29.27, 28.75 (C-B and C-E), 26.44, 25.51 (C-C and C-D), 21.13 (Ac); ESI HRMS (*m/z*): Calcd for $C_{35}H_{43}N_3O_7Na$ 640.2993, found 640.2992.

6'-Azidohexyl 3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (**65**).

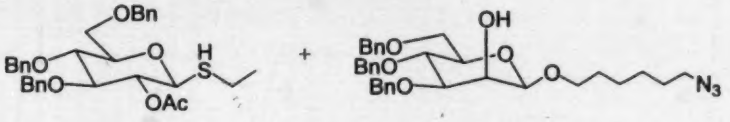


63 **65**

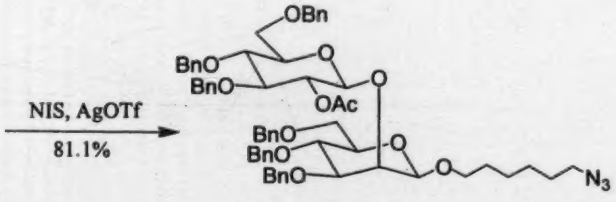
The acetylated mannopyranoside **63** (50mg, 0.080mmol) was transesterified with NaOMe (1.0M, 0.3mL) in MeOH (5mL) and heated at reflux for 1 h. The solution was neutralized with Amberlite IR 120 resin beads, filtered, and concentrated *in vacuo*. The residue was chromatographed with 8:1 toluene-ethyl acetate as eluant to yield compound **65** (44mg, 95%); $[\alpha]_D^{25}$ -29.4 (*c* 0.88, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ_H, 7.41-7.18 (m, 15H, aromatic), 4.89 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.78 (d, 1H, ^2J = 11.8 Hz, OCH_2Ph), 4.68 (d, 1H, ^2J = 11.9 Hz, OCH_2Ph), 4.62 (d, 1H, ^2J = 12.1 Hz, OCH_2Ph), 4.56 (d, 1H, ^2J = 11.9 Hz, OCH_2Ph), 4.54 (d, 1H, ^2J = 10.6 Hz, OCH_2Ph), 4.41 (d, 1H, J = 0.7 Hz, H-1), 4.11 (d, 1H, J = 2.9 Hz, H-2), 3.94 (ddd, 1H, J = 6.6, 6.6, 9.4 Hz, H-A1), 3.86 (dd, 1H, J = 9.3, 9.3 Hz, H-4), 3.78 (dd, 1H, J = 2.0, 10.8 Hz, H-6a), 3.71 (dd, 1H, J = 5.5, 10.8 Hz, H-6b), 3.57 (dd, 1H, J = 3.1, 9.0 Hz, H-3), 3.51 (ddd, 1H, J = 6.8, 6.8, 9.4 Hz, H-A2), 3.43 (ddd, 1H, J = 2.0, 5.4, 10.7 Hz, H-5), 3.26 (t, 2H, J = 6.9 Hz, H-F1, H-F2), 2.41 (broad s, 1H, OH), 1.70-1.47 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.45-1.34 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 138.30, 138.23, 137.84 ($\underline{C}_6H_5CH_2O$), 128.48, 128.36, 128.32, 128.08, 127.91, 127.85, 127.77, 127.72, 127.56 ($\underline{C}_6H_5CH_2O$), 99.78 (C-1, $^1J_{C\text{-}H}$ = 156.3 Hz), 81.62 (C-3), 75.33 (C-4), 73.17 ($O\underline{C}H_2Ph$), 74.32 (C-5), 73.52, 71.41 ($O\underline{C}H_2Ph$), 69.57 (C-

A), 69.33 (C-6), 68.37 (C-2), 51.39 (C-F), 29.40, 28.77 (C-B and C-E), 26.51, 25.64 (C-C and C-D); ESI HRMS (*m/z*): Calcd for $C_{33}H_{41}N_3O_6Na$ 598.2888, found 598.2885.

6′-Azidohexyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (**66**).



67 **65**

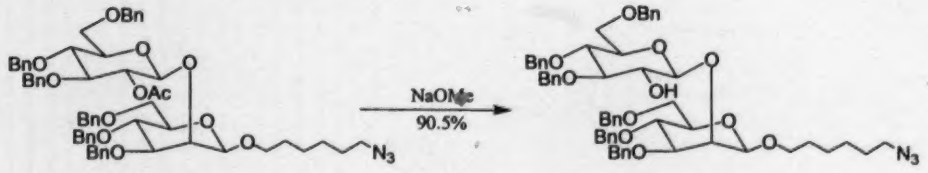


66

A mixture of thioglycoside **67** (0.119g, 0.215mmol), acceptor **65** (0.072g, 0.12mmol), 4 Å molecular sieves (0.143g), and CH_3CN (1mL) was stirred under argon at room temperature for 1 h. After cooling to –30 °C, *N*-iodosuccinimide (0.0462g, 0.205mmol) was added and the solution stirred for 10 min before the addition of silver triflate (0.0076g, 0.124mmol). After 45 min, the reaction was neutralized with Et_3N, diluted with CH_2Cl_2, and washed successively with sat'd $Na_2S_2O_3$, H_2O, and sat'd brine. The organic layer was dried over Na_2SO_4, filtered, concentrated to dryness, and purified by chromatography using 20:1 toluene-ethyl acetate as the eluant. Compound **66** was obtained as a syrup (0.106g, 81%); $[\alpha]_D^{25}$ -1.1 (*c* 0.28, $CHCl_3$); ^1H NMR

(500 MHz, $CDCl_3$): δ_H, 7.39-7.16 (m, 30H, aromatic), 5.12 (dd, 1H, J = 8.1, 9.6 Hz, H-2'), 4.92 (d, 1H, ^2J = 10.9 Hz, OCH_2Ph), 4.88 (d, 1H, ^2J = 12.4 Hz, OCH_2Ph), 4.87 (d, 1H, J = 8.2 Hz, H-1'), 4.82 (d, 1H, ^2J = 10.9 Hz, OCH_2Ph), 4.78 (d, 1H, ^2J = 11.4 Hz, OCH_2Ph), 4.74 (d, 1H, ^2J = 11.6 Hz, OCH_2Ph), 4.55 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.55 (s, 2H, 2 × OCH_2Ph), 4.51 (d, 1H, ^2J = 11.9 Hz, OCH_2Ph), 4.45 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.29 (d, 1H, $J \leq$ 1 Hz, H-1), 4.24 (d, 1H, J = 3.0 Hz, H-2), 3.86 (ddd, 1H, J = 6.5, 6.5, 9.3 Hz, H-A1), 3.76 (dd, 1H, J = 1.8, 10.8 Hz, H-6a'), 3.75 (dd, 1H, J = 1.8, 10.6 Hz, H-6a), 3.71 (dd, 1H, J = 8.6, 9.5 Hz, H-3'), 3.65 (dd, 1H, J = 6.1, 10.7 Hz, H-6b), 3.62-3.53 (m, 4H, H-4, H-5, H-4', H-6b'), 3.47 (dd 1H, J = 3.1, 9.2 Hz, H-3), 3.44-3.37 (m, 2H, H-5', H-A2), 3.27 (t, 2H, J = 6.9 Hz, H-F1, H-F2), 1.94 (s, 3H, OAc), 1.66-1.50 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.50-1.34 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 169.76 (C=O), 138.53, 138.43, 138.18, 138.01, 137.89 ($\underline{C}_6H_5CH_2O$), 128.42, 128.36, 128.34, 128.32, 128.27, 128.26, 128.14, 128.04, 127.73, 127.64, 127.61, 127.51, 127.49 ($\underline{C}_6H_5CH_2O$), 101.03 (C-1', $^1J_{C\text{-}H}$ = 163.5 Hz), 100.58 (C-1, $^1J_{C\text{-}H}$ = 153.4 Hz), 83.23 (C-3'), 80.07 (C-3), 78.09 (H-4'), 75.52 (C-5'), 75.23 ($O\underline{C}H_2Ph$), 75.07 ($O\underline{C}H_2Ph$), 74.94, 74.79 (C-4 or C-5), 74.74, 73.56 ($O\underline{C}H_2Ph$), 73.34 (C-2'), 73.17 ($O\underline{C}H_2Ph$), 72.22 (C-2), 70.44 (C-6), 69.92 (C-6'), 69.76 ($O\underline{C}H_2Ph$), 60.05 (C-A), 68.86 (C-6), 51.39 (C-F), 29.58, 28.88 (C-B and C-E), 26.57, 25.82 (C-C and C-D), 21.09 (Ac); ESI HRMS (*m/z*): Calcd for $C_{62}H_{71}N_3O_{12}Na$ 1072.4930, found 1072.4928.

6'-Azidohexyl 3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (**68**).

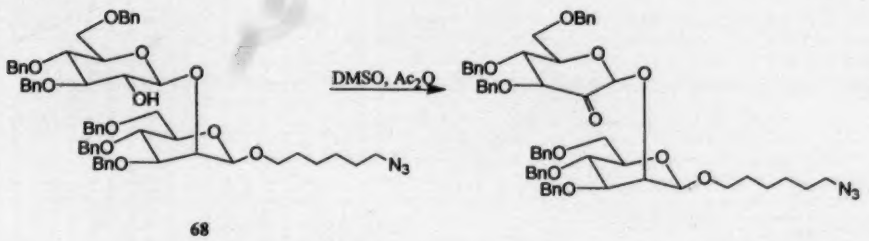


66 **68**

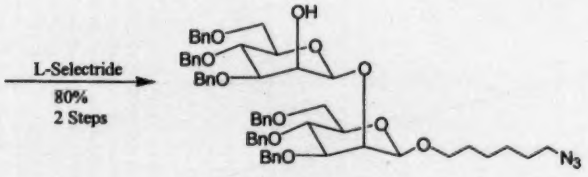
Disaccharide **66** (0.167g, 0.159mmol) in dry MeOH (30mL) was treated with NaOMe (3.5 M ,1.2mL) and heated at reflux for 1 h. The reaction solution was neutralized with Amberlite IR 120 acidic resin beads, filtered, and concentrated *in vacuo*. Flash silica gel chromatography of the residue using a gradient of 20:1 to 10:1 toluene-ethyl acetate afforded compound **68** (0.145g, 91%); $[\alpha]_D^{25}$ -22.3 (*c* 0.35, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$): δ_H, 7.44-7.15 (m, 30H, aromatic), 5.06 (d, 1H, ^2J = 11.2 Hz, OCH_2Ph), 4.93 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.90 (d, 1H, ^2J = 12.0 Hz, OCH_2Ph), 4.87 (d, 1H, ^2J = 10.9 Hz, OCH_2Ph), 4.79 (d, 1H, ^2J = 11.2 Hz, OCH_2Ph), 4.67 (d, 1H, J = 7.8 Hz, H-1'), 4.63 (d, 1H, ^2J = 12.1 Hz, OCH_2Ph), 4.56 (d, 1H, ^2J = 12.1 Hz, OCH_2Ph), 4.55 (d, 1H, ^2J = 12.0 Hz, OCH_2Ph), 4.52 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.49 (d, 2H, ^2J = 10.8 Hz, 2 × OCH_2Ph), 4.47 (d, 1H, ^2J = 12.0 Hz, OCH_2Ph), 4.37 (d, 1H, $J \leq 1$ Hz, H-1), 4.25 (d, 1H, J = 3.2 Hz, H-2), 3.93 (ddd, 1H, J = 6.6, 6.6, 9.4 Hz, H-A1), 3.88 (dd, 1H, J = 9.6, 9.6 Hz, H-4), 3.77 (dd, 1H, J = 1.9, 11.0 Hz, H-6a), 3.75-3.69 (m, 3H, H-6, H-2', H-6a'), 3.68-3.61 (m, 2H, H-3', H-6b'), 3.58-3.49 (m, 3H, H-3, H-4', H-5'), 3.46 (ddd, 1H, J = 6.5, 6.5, 9.2 Hz, H-A2), 3.41 (ddd, 1H, J = 1.8, 4.9, 9.7 Hz, H-5), 3.24 (t,

2H, J = 6.9 Hz, H-F1, H-F2), 1.68-1.55 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.46-1.34 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 138.07, 138.46, 138.35, 138.21, 138.12, 138.08 ($\underline{C}_6H_5CH_2O$), 128.51-127.39 ($\underline{C}_6H_5CH_2O$), 128.35, 128.33, 128.31, 128.26, 128.11, 128.08, 128.04, 127.99, 127.77, 127.70, 127.65, 127.61, 127.57, 127.54, 127.53, 127.42 ($\underline{C}_6H_5CH_2O$), 104.16 (C-1', $^1J_{C\text{-}H}$ = 160.9 Hz), 100.44 (C-1, $^1J_{C\text{-}H}$ = 154.5 Hz), 85.33 (C-3'), 80.29 (C-3'), 77.22 (C-4'), 75.70 (C-5), 75.39 (2C, C-2' and C-5'), 75.26 (O$\underline{C}H_2$Ph), 75.05 (O$\underline{C}H_2$Ph), 74.81 (C-2), 74.67 (O$\underline{C}H_2$Ph), 74.42 (C-4), 73.44, 73.41, 70.32 (O$\underline{C}H_2$Ph), 69.81 (C-6'), 69.59 (C-A), 69.34 (C-6), 51.37 (C-F), 29.40, 28.81 (C-B and C-E), 26.46, 25.60 (C-C and C-D); ESI HRMS (*m/z*): Calcd for $C_{60}H_{69}N_3O_{11}Na$ 1030.4824, found 1030.4822.

6'-Azidohexyl 3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (**69**).



68

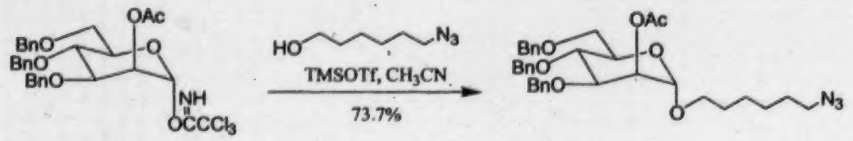


69

Disaccharide **68** (31mg, 0.0303mmol) was dissolved in dry DMSO (1.5mL). Acetic anhydride (0.73mL, 7.9mmol) was added and the solution stirred under argon at room temperature overnight. The reaction mixture was diluted with EtOAc and washed with H_2O and saturated brine. The organic layer was dried over $MgSO_4$, filtered, concentrated under reduced pressure and dried under high vacuum for 2.5 h. The residue was then taken up in anhydrous THF (0.5mL) and cooled to –20 °C under argon. L-Selectride (1.2mL, 1.2mmol) was added dropwise and after 30 min the reaction was quenched with MeOH. The solution was diluted with CH_2Cl_2 and successively washed with aqueous 6% H_2O_2, 1M NaOH, H_2O, and saturated brine. The organic extract was dried over a combination of $MgSO_4$ and Na_2SO_4, filtered, concentrated under vacuum, and purified by chromatography using a gradient of 8:1 to 6:1 toluene-ethyl acetate. Compound **69** was obtained as a syrup (24mg, 80%); ^1H

NMR (500 MHz, $CDCl_3$): δ_H, 7.42-7.12 (m, 30H, aromatic), 4.95 (d, 1H, ^2J = 10.9 Hz, OCH_2Ph), 4.94 (d, 1H, $J \leq 1$ Hz, H-1'), 4.92 (d, 1H, ^2J = 11.5 Hz, OCH_2Ph), 4.87 (d, 1H, ^2J = 10.7 Hz, OCH_2Ph), 4.83 (d, 1H, ^2J = 12.0 Hz, OCH_2Ph), 4.64 (d, 1H, ^2J = 11.6 Hz, OCH_2Ph), 4.61 (d, 1H, ^2J = 11.7 Hz, OCH_2Ph), 4.46 (d, 1H, ^2J = 10.2 Hz, OCH_2Ph), 4.45 (s, 1H, ^2J = 12.2 Hz, OCH_2Ph), 4.49 (d, 1H, J = 3.6 Hz, H-2), 4.47 (d, 1H, ^2J = 11.9 Hz, OCH_2Ph), 4.46 (d, 2H, ^2J = 11.5 Hz, 2 × OCH_2Ph), 4.44 (d, 1H, ^2J = 11.9 Hz, OCH_2Ph), 4.37 (d, 1H, $J \leq 1$ Hz, H-1), 4.33 (d, 1H, J = 3.1 Hz, H-2'), 3.94-3.88 (m, 2H, H-4', H-A1), 3.80-3.73 (m, 3H, H-4, H-6a, H-6a'), 3.70 (dd, 1H, J = 5.7, 10.8 Hz, H-6b), 3.60 (dd, 1H, J = 6.4, 10.9 Hz, H-6b'), 3.58-3.53 (m, 2H, H-3, H-3'), 3.51-3.46 (m, 1H, H-5'), 3.44-3.38 (m, 2H, H-5, H-A2), 3.22 (t, 2H, J = 6.9 Hz, H-F1, H- F2), 1.70 (broad s, 1H, OH), 1.61-1.50 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.40-1.24 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 138.36, 138.84, 138.29, 138.15 (2 × C), 138.08 ($\underline{C}_6H_5CH_2O$), 128.37, 128.34, 128.30, 128.27, 128.17, 128.12, 127.87, 127.68, 127.64, 127.59, 127.58, 127.53 ($\underline{C}_6H_5CH_2O$), 101.09 (C-1, $^1J_{C\text{-}H}$ = 153.9 Hz), 99.20 (C-1', $^1J_{C\text{-}H}$ = 162.3 Hz), 71.51, 80.40 (C-3 and C-3'), 75.59 (C-5'), 75.10 (2 × $O\underline{C}H_2Ph$), 74.43 (C-4'), 74.14 (C-4), 73.46, 73.35, 70.74 ($O\underline{C}H_2Ph$), 70.55 (C-2 and C-5), 70.08 ($O\underline{C}H_2Ph$), 69.99 (C-6), 69.95 (C-6'), 69.50 (C-A), 67.68 (C-2'), 51.33 (C-F), 29.44, 28.77 (C-B and C-E), 26.41, 25.56 (C-C and C-D); ESI HRMS (*m/z*): Calcd for $C_{60}H_{69}N_3O_{11}Na$ 1030.4824, found 1030.4822.

6'-Azidohexyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**71**).

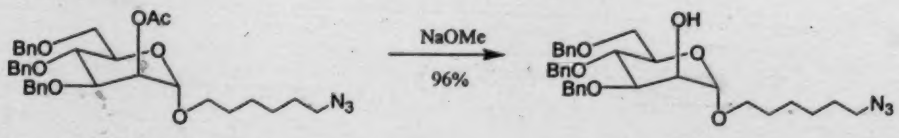


70 **71**

Trichloroacetimidate mannopyranosyl donor **70** (0.800g, 1.26mmol) and 6-azido-1-hexanol (0.360g, 2.51mmol) were dried together under vacuum for 1.5 h before dissolution in dry CH_3CN (4.5mL). 4Å Molecular sieves (0.375g) and a stir bar were previously flame dried and left under vacuum for 2 h before addition to the solution. The mixture was stirred for 1 h under argon at room temperature before being cooled to 0 °C. After 15 min, TMSOTf (22μL, 0.12mmol) was added dropwise. After 5 min, the reaction was allowed to warm up to room temperature and was further stirred for 1.5 h before being neutralized with Et_3N, concentrated to dryness, and purified by column chromatography using 20:1 toluene-ethyl acetate as eluant. Compound **71** was obtained as a syrup (0.572g, 73%); $[\alpha]_D^{25}$ +21.7 (*c* 1.08, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H, 7.38-7.14 (m, 15H, aromatic), 5.35 (dd, 1H, J = 1.8, 3.3 Hz, H-2), 4.86 (d, 1H, ^2J = 10.7 Hz, OCH_2Ph), 4.82 (d, 1H, J = 1.7 Hz, H-1), 4.71 (d, 1H, ^2J = 11.1 Hz, OCH_2Ph), 4.68 (d, 1H ^2J = 12.1 Hz, OCH_2Ph), 4.55 (s, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.53 (s, 1H, ^2J = 11.7 Hz, OCH_2Ph), 4.48 (s, 1H, ^2J = 10.7 Hz, OCH_2Ph), 3.98 (dd, 1H, J = 3.4, 9.3 Hz, H-3), 3.88 (dd, 1H, $J \approx$ 9.5 Hz, H-4), 3.83-3.76 (m, 2H, H-6a, H-5), 3.71 (m, 1H, H-6b), 3.67 (ddd, 1H, J = 6.4, 6.4, 9.5 Hz, H-A1), 3.40 (ddd, 1H, J = 6.4, 6.4, 9.6 Hz, H-

A2), 3.25 (t, 2H, J = 7.0 Hz, H-F1, H-F2), 2.15 (s, 3H, OAc), 1.60-1.46 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.38-1.24 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 170.53 (C=O), 138.35, 138.24, 137.99 ($\underline{C}_6H_5CH_2O$), 128.37,128.32, 128.29, 128.04, 127.94, 127.75, 127.71, 127.65, 127.57 ($\underline{C}_6H_5CH_2O$), 97.74 (C-1, $^1J_{C\text{-}H}$ = 170.0 Hz), 78.26 (C-3), 75.23 ($O\underline{C}H_2Ph$), 74.40 (C-4), 73.44, 71.78 ($O\underline{C}H_2Ph$), 71.39 (C-5), 68.95 (C-6), 68.87 (C-2), 67.72 (C-A), 51.37 (C-F), 29.24, 28.74 (C-B and C-E), 26.49, 25.72 (C-C and C-D), 21.14 (Ac); ESI HRMS (*m/z*): Calcd for $C_{35}H_{43}N_3O_7Na$ 640.2993, found 640.2991.

6′-Azidohexyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**72**).

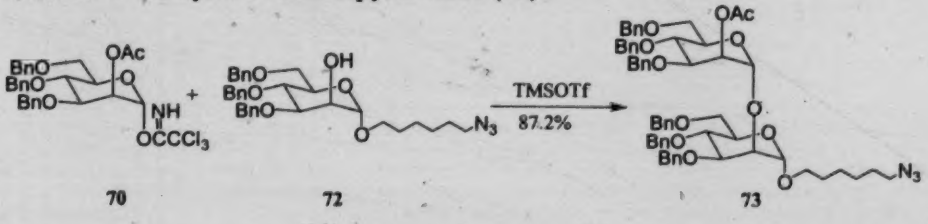


71 72

The acetylated mannopyranoside **71** (83 mg, 0.14mmol) was treated with NaOMe (1.0M, 28μL) in MeOH (1.5mL) and heated at reflux for 1 h. The reaction solution was neutralized with Amberlite IR 120 resin beads, filtered and concentrated to dryness. The filtrate was taken up in EtOAc and washed successively with H_2O and sat'd brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was chromatographed with 3:1 hexanes-ethyl acetate as the eluant to yield compound **72** (75mg, 96%); $[\alpha]_D^{25}$ +49.6 (*c* 0.13, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H, 7.40-7.16 (m, 15H, aromatic), 4.90 (d, 1H, J = 1.6 Hz, H-1), 4.83

(d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.73 (d, 1H, ^2J = 11.5 Hz, OCH_2Ph), 4.70 (d, 1H, ^2J = 11.4 Hz, OCH_2Ph), 4.66 (d, 1H, ^2J = 12.2 Hz, OCH_2Ph), 4.55 (d, 1H, ^2J = 12.1 Hz, OCH_2Ph), 4.52 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.04 (dd, 1H, J = 1.9, 3.1 Hz, H-2), 3.89 (dd, 1H, J = 3.2, 9.0 Hz, H-3), 3.85 (dd, 1H, J = 9.3, 9.3 Hz, H-4), 3.80-3.66 (m, 4H, H-5, H-6a, H-6b, H-A1), 3.43 (ddd, 1H, J = 6.5, 6.5, 8.7 Hz, H-A2), 3.25(t, 2H, J = 7.0 Hz, H-F1, H-F2), 2.25 (broad s, 1H, OH), 1.64-1.53 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.46-1.31 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 138.28 (2 × C), 137.96 ($\underline{C}_6H_5CH_2O$), 128.52, 128.36, 128.31, 127.99, 127.90, 127.81, 127.70, 127.55 ($\underline{C}_6H_5CH_2O$), 99.17 (C-1, $^1J_{C\text{-}H}$ = 168.7 Hz), 80.31 (C-3), 75.19 ($O\underline{C}H_2Ph$), 74.38 (C-4), 73.46, 71.98 ($O\underline{C}H_2Ph$), 71.08 (C-5), 69.02 (C-6), 68.45 (C-2), 67.51 (C-A), 51.37 (C-F), 29.27, 28.76 (C-B and C-E), 26.50, 25.75 (C-C and C-D); ESI HRMS (*m/z*): Calcd for $C_{33}H_{41}O_6Na$ 598.2888, found 598.2888.

6′-Azidohexyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**73**).



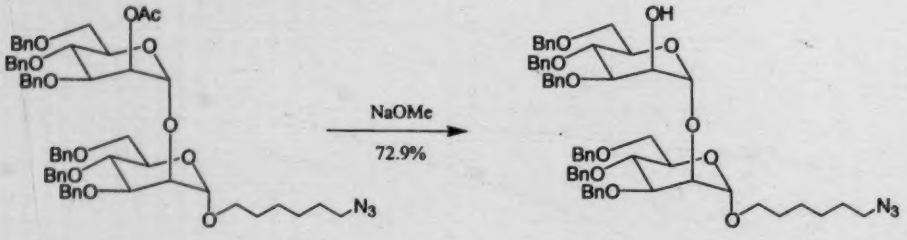
70 **72** **73**

Trichloroacetimidate mannopyranosyl donor **70** (0.748g,1.17mmol) and azidoglycoside **72** (0.300g, 0.52mmol) were dried together under vacuum for 2 h before dissolution in dry CH_3CN (4.5mL). 4Å Molecular sieves (0.341g) and a stir bar were that were previously flame dried and left under vacuum for

2 h were added to the solution. The mixture was stirred for 1 h under argon at room temperature before being cooled to 0 °C for 5 min. TMSOTf (30μL, 0.166mmol) was added dropwise and after 5 min, the reaction was allowed to warm up to room temperature and stirred for 20 min. Additional TMSOTf (20μL, 0.111mmol) was then added to re-acidify the reaction solution which was further stirred for 30 min before being neutralized with Et_3N, concentrated to dryness, and purified by column chromatography in 30:1 toluene-ethyl acetate. Compound **73** was obtained as a syrup (0.477g, 87%); $[\alpha]_D^{25}$ +26.4 (*c* 0.25, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H, 7.37-7.12 (m, 30H, aromatic), 5.54 (dd, 1H, J = 1.9, 3.3 Hz, H-2'), 5.09 (d, 1H, J = 1.8 Hz, H-1'), 4.88-4.83 (m, 3H, H-1, 2 × OCH_2Ph), 4.69 (s, 2H, 2 × OCH_2Ph), 4.66 (d, 1H, ^2J = 10.9 Hz, OCH_2Ph), 4.65 (d, 1H, ^2J = 12.2 Hz, OCH_2Ph), 4.56 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.54 (d, 1H, ^2J = 12.3 Hz, OCH_2Ph), 4.49 (d, 1H, ^2J = 13.1 Hz, OCH_2Ph), 4.47 (d, 1H, ^2J = 11.3 Hz, OCH_2Ph), 4.41 (d, 1H, ^2J = 10.9 Hz, OCH_2Ph), 4.01-3.97 (m, 2H, H-2, H-3'), 3.96 (ddd, 1H, J = 1.8, 4.8, 9.9 Hz, H-5'), 3.91 (dd, 1H, J = 2.1, 9.3 Hz, H-3), 3.85-3.68 (m, 7H, H-4, H-5, H-6a, H-6b, H-4', H-6a', H-6b'), 3.59 (ddd, 1H, J = 6.7, 6.7, 9.6 Hz, H-A1), 3.26 (ddd, 1H, J = 6.4, 6.4, 9.6 Hz, H-A2), 3.23 (t, 2H, J = 7.0 Hz, H-F1, H-F2), 2.12 (s, 3H, OAc), 1.64-1.52 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.44-1.30 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 170.10 (C=O), 138.55, 138.48, 138.41, 138.39, 138.07, 138.02 ($\underline{C}_6H_5CH_2O$), 128.51-127.39 ($\underline{C}_6H_5CH_2O$), 99.55 (C-1', $^1J_{C\text{-}H}$ = 172.2 Hz), 98.67 (C-1', $^1J_{C\text{-}H}$ = 170.1 Hz), 79.74 (C-3), 78.14 (C-3'), 75.19 ($O\underline{C}H_2Ph$), 75.06 ($O\underline{C}H_2Ph$),

75.00 (C-2), 74.72 (C-4 or C-4'), 74.42 (C-4 or C-4'), 73.34, 73.30, 72.03, 71.91 ($O\underline{C}H_2Ph$), 71.86 (C-5 or C-5'), 71.81 (C-5 or C-5'), 69.35 (C-6 or C-6'), 69.17 (C-6 or C-6'), 68.77 (C-2'), 67.47 (C-A), 51.37 (C-F), 29.30 (C-B or C-E), 28.75 (C-B or C-E), 26.50 (C-C or C-D), 25.72 (C-C or C-D), 21.13 (Ac); ESI HRMS (*m/z*): Calcd for $C_{62}H_{71}N_3O_{12}Na$ 1072.4930, found 1072.4929.

6'-Azidohexyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**74**).

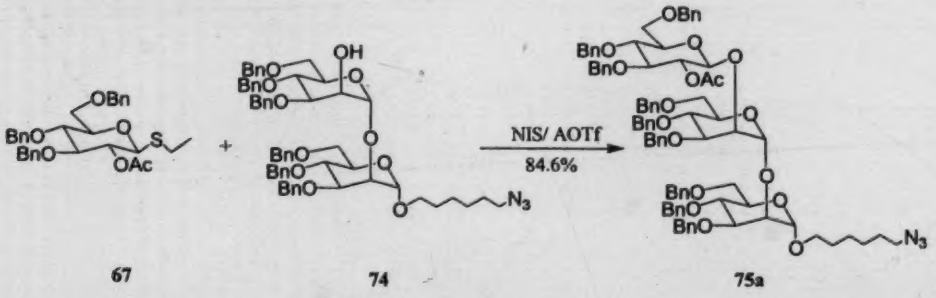


73 **74**

Compound **73** (62 mg, 0.059mmol) in dry MeOH (2mL) was treated with NaOMe (1M, 20μL) and heated at reflux for 1 h. The solution was neutralized with Amberlite IR 120 acidic resin beads, diluted with EtOAc, and successively washed with H_2O, and saturated brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. Silica gel chromatography of the residue with 6:1 toluene-ethyl acetate as the eluant afforded compound **74** (43mg, 73%); $[\alpha]_D^{25}$ +37.5 (*c* 0.99, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ_H, 7.37-7.16 (m, 30H, aromatic), 5.15 (d, 1H, J = 1.5 Hz, H-1'), 4.90 (d, 1H, J = 1.7 Hz, H-1), 4.84 (d, 1H, ^2J = 10.4 Hz, OCH_2Ph), 4.81 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.70 (d, 1H, ^2J = 11.6, OCH_2Ph), 4.69 (d,

1H, ^2J = 12.2 Hz, OCH_2Ph), 4.66 (d, 1H, ^2J = 11.6 Hz, OCH_2Ph), 4.63 (d, 1H, ^2J = 12.2 Hz, OCH_2Ph), 4.60 (d, 1H, ^2J = 11.4 Hz, OCH_2Ph), 4.57 (d, 1H, ^2J = 11.0 Hz, OCH_2Ph), 4.55 (d, 2H, ^2J = 10.4 Hz, 2 × OCH_2Ph), 4.52 (d, 1H, J = 12.1 Hz, OCH_2Ph), 4.51 (d, 1H, ^2J = 11.0 Hz, OCH_2Ph), 4.13 (dd, 1H, J = 1.9, 3.2 Hz, H-2'), 4.03 (dd, 1H, J = 2.6, 2.6 Hz, H-2), 3.97 (ddd, 1H, J = 2.4, 4.5, 9.7 Hz, H-5'), 3.92 (dd, 1H, J = 2.9, 9.3 Hz, H-3), 3.88 (dd, 1H, J = 3.2, 9.1 Hz, H-3'), 3.86-3.77 (m, 3H, H-4, H-4', H-6a'), 3.77-3.68 (m, 4H, H-5, H-6a, H-6b, H-6b'), 3.58 (ddd, 1H, J = 6.7, 6.7, 9.6 Hz, H-A1), 3.26 (ddd, 1H, J = 6.4, 6.4, 9.6 Hz, H-A2), 3.23 (t, 2H, J = 7.0 Hz, H-F1, H-F2), 1.82 (broad s, 1H, OH), 1.61-1.42 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.38-1.22 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 138.67, 138.50, 138.42, 138.36, 138.33, 138.02 ($\underline{C}_6H_5CH_2O$), 128.44, 128.41, 128.32, 128.28, 128.26, 128.25, 127.97, 127.85, 127.82, 127.75, 127.69, 127.65, 127.61, 127.57, 127.48, 127.42, 127.34 ($\underline{C}_6H_5CH_2O$), 101.10 (C-1', $^1J_{C\text{-}H}$ = 170.9 Hz), 98.82 (C-1', $^1J_{C\text{-}H}$ = 170.0 Hz), 80.03 (C-3'), 79.83 (C-3), 75.12 ($O\underline{C}H_2Ph$), 75.11 (C-2), 74.98 ($O\underline{C}H_2Ph$), 74.93 (C-4'), 74.53 (C-4), 73.39, 73.32, 72.25, 72.15 ($O\underline{C}H_2Ph$), 71.96 (C-5), 71.60 (C-5'), 69.45 (C-6'), 69.36 (C-6), 68.58 (C-2'), 67.49 (C-A), 51.38 (C-F), 29.32 (C-B or C-E), 28.74 (C-B or C-E), 26.49 (C-C or C-D), 25.71 (C-C or C-D); ESI HRMS (*m/z*): Calcd for $C_{60}H_{69}N_3O_{11}Na$ 1030.4824, found 1030.4827.

6'-Azidohexyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**75a**).



67 74 75a

The thioglycoside **67** (79mg, 0.15mmol) and acceptor **74** (81mg, 0.080mmol) were dried together under vacuum before dissolution in dry CH_2Cl (1.5mL). 4Å Molecular sieves (0.255g) and a stir bar were previously flame dried and kept under vacuum before addition to the solution. The mixture was stirred for 1 h under argon at room temperature before being cooled to -30 °C. *N*-Iodosuccinimide (0.031g, 0.14mmol) was added to the solution followed 10 min later by silver triflate (5.3mg, 0.021mmol). After being stirred for 35 min, the reaction mixture was allowed to warm up to 10 °C and then quenched with Et_3N. The solution was concentrated and the residue purified by column chromatography using 20:1 toluene-ethyl acetate as eluent. Compound **75a** was obtained as a syrup (0.101g, 85%); ^1H NMR (600 MHz, $CDCl_3$): δ_H, 7.38-7.14 (m, 45H, aromatic), 5.05 (dd, 1H, J = 8.1, 9.0 Hz, H-2''), 4.98 (d, 1H, J = 1.8 Hz, H-1'), 4.88 (s, 1H, H-1), 4.88-4.42 (m, 17H, 17 × OCH_2Ph), 4.38 (d, 1H, ^2J = 11.0 Hz, OCH_2Ph), 4.25 (broad doublet, 1H, $J \approx$ 8.1 Hz, H-1''), 4.16 (dd, 1H, J = <1, 2.6 Hz, H-2'), 4.04 (dd, 1H, J = <1, 2.5 Hz, H-2), 3.93-3.87 (m, 3H, H-3, H-3', H-6a'), 3.79-3.66 (m, 6H, H-4, H-5, H-6a, H-6b,

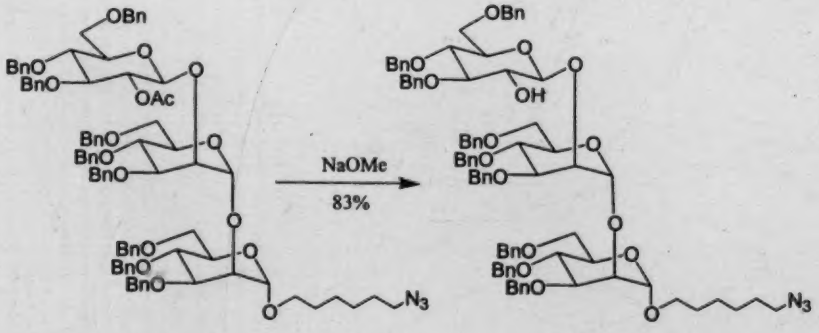
H-5', H-6b'), 3.65-3.52 (m, 5H, H-4', H-4'', H-6a'', H-6b'', H-A1), 3.49 (dd, 1H, J = 9.3, 9.3 Hz, H-3''), 3.34 (m, 1H, H-5''), 3.25-3.18 (m, 3H, H-A2, H-F1, H-F2), 1.9 (s, 3H, OAc), 1.58-1.40 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.38-1.20 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 169.35 (C=O), 169.35 (C=O), 138.58, 138.55, 138.51, 138.45, 138.40, 138.31, 138.10, 137.96 ($\underline{C}_6H_5CH_2O$), 128.60, 128.38, 128.35, 128.28, 128.26, 128.24, 128.23, 128.15, 127.90, 127.88, 127.82, 127.77, 127.73, 127.71, 127.68, 127.65, 127.59, 127.53, 127.51, 127.45, 127.41, 127.38 ($\underline{C}_6H_5CH_2O$), 99.74, 99.39, 98.82 (C-1, C-1', C-1''), 82.65 (C-3''), 80.236 (C-3 or C-3'), 77.92, 77.55 (C-3 or C-3'), 75.07, 75.04, 74.80, 74.73, 74.60, 74.43, 74.13, 73.53, 73.39, 72.96, 72.78, 72.60, 71.88, 71.04, 70.40, 69.42, 67.42 (C-A), 51.36 (C-F), 29.30, 28.73 (C-B and C-E), 26.49, 25.69 (C-C and C-D), 20.97 (Ac); ESI HRMS (*m/z*): Calcd for $C_{89}H_{99}N_3O_{17}Na$ 1504.6867, found 1504.6868.

Tentative Assignment:

^{13}C NMR (125 MHz, $CDCl_3$): δ_C,169.35 (C=O), 138.58, 138.55, 138.51, 138.45, 138.40,138.31, 138.10, 137.96 ($\underline{C}_6H_5CH_2O$), 128.60, 128.38, 128.35, 128.28, 128.26, 128.24, 128.23, 128.15, 127.90, 127.88, 127.82, 127.77, 127.73, 127.71, 127.68, 127.65, 127.59, 127.53, 127.51, 127.45, 127.41, 127.38 ($\underline{C}_6H_5CH_2O$), 99.74 (C-1'', $^1J_{C\text{-}H}$ = 161.8 Hz), 99.39 (C-1', $^1J_{C\text{-}H}$ = 170.7 Hz), 98.82(C-1, $^1J_{C\text{-}H}$ = 170.7 Hz), 82.65 (C-3''), 80.24 (C-3 or C-3'), 77.92 (C-4' or C-4''), 77.55 (C-3 or C-3'), 75.07 (C-5''), 75.04 (C-4), 74.80, 74.73, 74.60 ($O\underline{C}H_2Ph$), 74.43 (C-2), 74.13 (C-2'), 73.53, 73.39, 72.96

($O\underline{C}H_2Ph$), 72.78 (C-2''), 72.60 ($O\underline{C}H_2Ph$), 71.88 (C-5 and C-5'), 71.04, 70.40 ($O\underline{C}H_2Ph$), 69.42 (C-6, C-6', C-6''), 67.42 (C-A), 51.36 (C-F), 29.30, 28.73 (C-B and C-E), 26.49, 25.69 (C-C and C-D), 20.97 (Ac).

6'-Azidohexyl 3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**75b**).

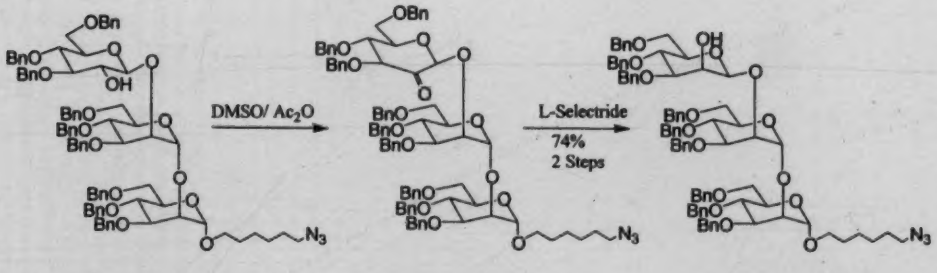


75a **75b**

To acetylated **75a** (92 mg, 0.062mmol) dissolved in dry MeOH (20mL) was added NaOMe (3.5M, 0.8mL). The solution was heated at reflux for 1 h. The reaction mixture was neutralized with Amberlite IR 120 H^+ resin beads and the solution concentrated *in vacuo*. Flash silica gel chromatography of the residue with 8:1 toluene-ethyl acetate as the eluant afforded compound **75b** (74mg, 83%); ^{1}H NMR (600 MHz, $CDCl_3$): δ_H, 7.41-7.13 (m, 45H, aromatic), 5.10 (d, 1H, J = 3.2 Hz, H-1'), 4.94 (d, 1H, ^2J = 11.3 Hz, OCH_2Ph), 4.88 (d, 1H, J = 1.6 Hz, H-1), 4.86 (d, 1H, ^2J = 10.9 Hz, OCH_2Ph), 4.75 (d, 1H, ^2J = 11.7 Hz, OCH_2Ph), 4.72-4.42 (m, 14H, OCH_2Ph), 4.32 (broad doublet, 1H, J = 10.8 Hz, H-1''), 4.21 (dd, 1H, J = 3.6, 3.6 Hz, H-2''), 4.11 (dd, 1H, J = 3.0, 3.0 Hz, H-2'), 3.99 (m, 1H, H-2), 3.98 (dd, 1H, J = 2.9, 10.7 Hz, H-3'), 3.91

(ddd, 1H, J = 2.1, 4.4, 9.3 Hz, H-5'), 3.88 (dd, 1H, J = 3.1, 8.9 Hz, H-3), 3.84 (dd, 1H, J = 7.5, 7.5 Hz, H-4'), 3.78-3.61 (m, 7H, H-4, H-5, H-6a, H-6b, H-6a', H-6b', H-6a''), 3.60-3.53 (m, 2H, H-6b'', H-A1), 3.48 (m, 1H, H-3''), 3.44 (dd, 1H, J = 9.4, 9.4 Hz, H-4''), 3.34 (ddd, 1H, J = 1.5, 5.1, 9.6 Hz, H-5''), 3.23 (m, 1H, H-A2), 3.22 (t, 2H, J = 6.9 Hz, H-F1, H-F2), 1.60-1.44 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.37-1.22 (m, 4H, H-C1, H-C2, H-D1, H-D2); ESI HRMS (*m/z*): Calcd for $C_{87}H_{97}N_3O_{16}Na$ 1462.6761, found 1462.6761.

6'-Azidohexyl 3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**76**).



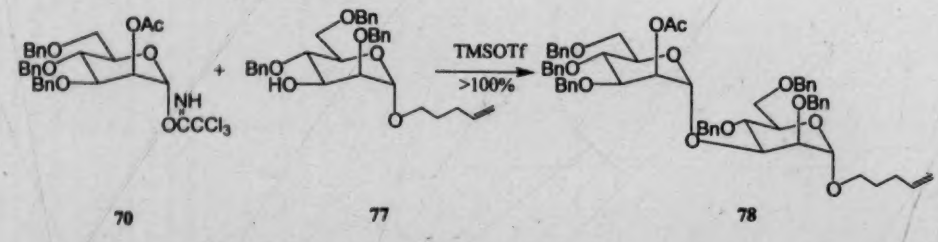
75b **76**

Trisaccharide **75b** (63mg, 0.044mmol) was dissolved in dry DMSO (3mL). Acetic anhydride (1.5mL) was added and the solution stirred under argon at room temperature overnight. The reaction mixture was diluted with EtOAc and washed with H_2O and saturated brine. The organic layer was dried over $MgSO_4$, filtered, concentrated under reduced pressure and dried under high vacuum for 2.5 h. The residue was taken up in anhydrous THF (0.5mL) and cooled to –20 °C under argon. L-Selectride (1.2mL, 1.2mmol) was added dropwise and after 30 min the reaction was quenched with MeOH. The

solution was diluted with CH_2Cl_2 and successively washed with aqueous 6% H_2O_2, 1M NaOH, H_2O, and saturated brine. The organic extract was dried over a combination of MgSO4 and Na_2SO_4, filtered, concentrated under vacuum, and purified by column chromatography using a gradient of 8:1 to 6:1 toluene-ethyl acetate as eluant. Compound **76** was obtained as a syrup (47mg, 74%); ^{1}H NMR (600 MHz, $CDCl_3$): δ_H, 7.38-7.08(m, 45H, aromatic), 5.13 (d, 1H, J = 2.5 Hz, H-1'), 4.91 (d, 1H, ^2J = 11.2 Hz, OCH_2Ph), 4.87 (d, 1H, J = 1.7 Hz, H-1), 4.83 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.78 (d, 1H, ^2J = 11.4 Hz, OCH_2Ph), 4.75 (d, 1H, ^2J = 11.0 Hz, OCH_2Ph), 4.68 (d, 1H, ^2J = 12.4 Hz, OCH_2Ph), 4.66 (d, 1H, ^2J = 11.4 Hz, OCH_2Ph), 4.63 (d, 1H, ^2J = 12.3 Hz, OCH_2Ph), 4.62 (d, 1H, ^2J = 12.1 Hz, OCH_2Ph), 4.61 (d, 1H, ^2J = 11.2 Hz, OCH_2Ph), 4.55 (d, 1H, ^2J = 12.3 Hz, OCH_2Ph), 4.53 (d, 1H, ^2J = 11.4 Hz, OCH_2Ph), 4.51 (d, 1H, ^2J = 12.4 Hz, OCH_2Ph), 4.50 (d, 1H, ^2J = 10.6 Hz, OCH_2Ph), 4.49 (d, 1H, ^2J = 12.1 Hz, OCH_2Ph), 4.43 (d, 1H, ^2J = 11.4 Hz, OCH_2Ph), 4.42 (d, 1H, ^2J = 12.2 Hz, OCH_2Ph), 4.40 (dd, 1H, J = 3.0, 3.0 Hz, H-2'), 4.36 (d, 1H, $J \leq 1$ Hz, H-1''), 4.35 (d, 1H, J = 11.1 Hz, OCH_2Ph), 4.33 (d, 1H, ^2J = 12.1 Hz, OCH_2Ph), 4.07 (dd, 1H, J = 2.0, 2.9 Hz, H-2), 4.01 (d, 1H, J = 3.0 Hz, H-2''), 3.96-3.84 (m, 4H, H-3, H-3', H-5', H-4''), 3.80-3.63 (m, 8H, H-4, H-5, H-6a, H-6b, H-4', H6a', H-6b', H-6a''), 3.61-3.53 (m, 2H, H-6b'', H-A1), 3.33 (dd, 1H, J = 3.1, 8.9 Hz, H-3''), 3.27-3.17 (m, 4H, H-5'', H-A2, H-F1, H-F2), 1.66-1.44 (m, 5H, OH, H-B1, H-B2, H-E1, H-E2), 1.37-1.22 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C,138.63, 138.51, 138.47, 138.34, 138.30, 138.27, 138.21,

138.13, 138.06 ($\underline{C}_6H_5CH_2O$), 128.51, 128.40, 128.37, 128.34, 128.30, 128.29, 128.24, 128.23, 128.13, 128.02, 127.92, 127.85, 127.81, 127.76, 127.73, 127.69, 127.64, 127.54, 127.48, 127.44 ($\underline{C}_6H_5CH_2O$), 99.62 (C-1', $^1J_{C\text{-}H}$ = 170.1 Hz), 98.86 (C-1, $^1J_{C\text{-}H}$ = 170.1 Hz), 97.29 (C-1'', $^1J_{C\text{-}H}$ = 159.5 Hz), 80.97 (C-3''), 80.23 (C-3), 77.67 (C-3'), 75.22 (C-4 or C-4'), 75.18 ($O\underline{C}H_2Ph$), 75.03 (C-4 or C-4' and C-5''), 74.60 (C-2), 74.30 ($O\underline{C}H_2Ph$), 74.11 (C-4''), 73.39, 73.25, 73.21, 72.70 ($O\underline{C}H_2Ph$), 72.01 (C-2'), 71.82, 71.59 (C-5 and C-5'), 71.01, 70.77 ($O\underline{C}H_2Ph$), 69.59, 69.49, 69.39 (C-6, C-6', C-6''), 67.97 (C-2''), 67.43 (C-A), 51.34 (C-F), 29.30, 28.74 (C-B and C-E), 26.49, 25.71 (C-C, C-D).

Pentenyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**78**).

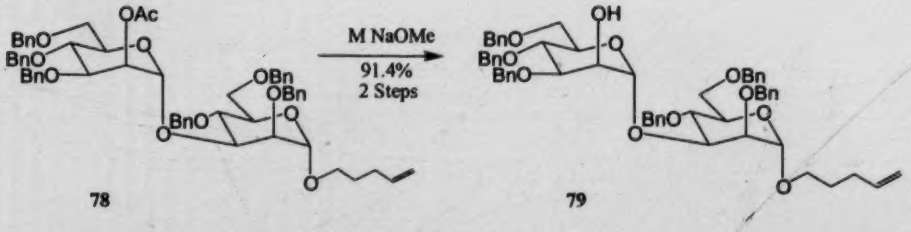


70 **77** **78**

Trichloroacetimidate **70** (0.157g, 0.246mmol) and **77** (0.06g, 0.12mmol) were dissolved in dry CH_3CN (4mL). Flame dried 4A molecular sieves (0.125g) were added and the mixture was stirred at room temperature for 1 h before being cooled to –30 °C and stirred for 10 more min. TMSOTf (15μL, 0.083mmol) was added and the reaction pH was monitored to ensure moderately acidic conditions. The reaction was warmed up slowly to 0 °C and monitored by TLC in 4:1 toluene-ethyl acetate. After 45 min, the reaction

mixture was neutralized with Et_3N and concentrated to dryness. The syrupy residue was purified by chromatography using 20:1 toluene-ethyl acetate as eluant to afford compound **78** which was contaminated with approximately 30% of the rearranged trichloroacetimidate **70** according to ^1H NMR. No further purification was carried out and acetylated trisaccharide **78** was taken on to the next step.

Pentenyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**79**).

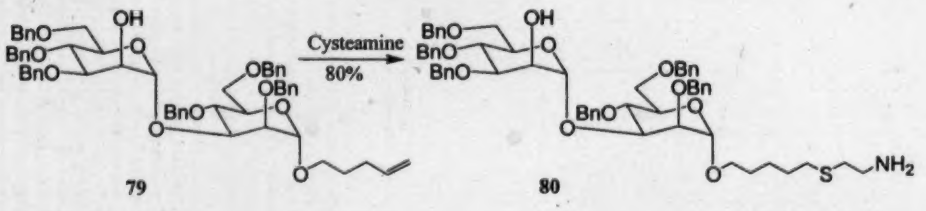


78 79

The acetylated disaccharide **78** (0.125g, 0.126mmol) was dissolved in freshly distilled MeOH (20mL). NaOMe (3.5M , 0.5mL,) was added dropwise to the solution and heated at reflux at 80 ^{0}C. After 1 h, all the starting material was consumed as seen by TLC in 4:1 toluene-ethyl acetate.The reaction was neutralized with Amberlite IR 120 H^+ resin beads and concentrated *in vacuo*. The residue was purified by chromatography using a gradient of 12:1 to 10:1 toluene-ethyl acetate as the eluant to afford **79** (0.103g, 91%) as a syrup; $[\alpha]_D^{25}$ +41.6 (*c* 0.80, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ_H, 7.38-7.17 (m, 30H, aromatic), 5.76 (ddt, 1H, J = 6.7, 10.3, 16.9 Hz, H-D), 5.24 (d, 1H, J = 1.5 Hz, H-1'), 4.98 (ddt, 1H, J = 1.6, 1.6, 17.1 Hz, H-E1), 4.94 (ddt, 1H, 1.3, 1.3, 10.2 Hz H-E2), 4.84 (d, 1H, ^2J = 10.8 Hz, OCH_2Ph), 4.83 (s, 1H, H-1), 4.68 (d, 1H,

^2J = 12.0 Hz, OCH_2Ph), 4.66 (d, 1H, ^2J = 13.2 Hz, OCH_2Ph), 4.65 (d, 1H, ^2J = 11.0 Hz, OCH_2Ph), 4.64 (d, 1H, ^2J = 13.2 Hz, OCH_2Ph), 4.63 (d, 1H, ^2J = 12.0 Hz, OCH_2Ph), 4.61 (d, 1H, ^2J = 12.0 Hz, OCH_2Ph), 4.59 (d, 1H, ^2J = 12.2 Hz, OCH_2Ph), 4.53 (d, 1H, ^2J = 12.0 Hz, OCH_2Ph), 4.51 (d, 1H, ^2J = 11.1 Hz, OCH_2Ph), 4.49 (d, 1H, ^2J = 11.9 Hz, OCH_2Ph), 4.47 (d, 1H, ^2J = 10.4 Hz, OCH_2Ph), 4.14 (dd, 1H, J = 3.0, 9.3 Hz, H-3), 4.00 (dd, 1H, J = 2.0, 3.0 Hz, H-2'), 3.98 (dd, 1H, J = 9.4, 9.4 Hz, H-4), 3.93 (ddd, 1H, J = 3.2, 3.2, 9.9 Hz, H-5'), 3.90 (dd, 1H, J = 3.1, 9.0 Hz, H-3'), 3.84 (dd, 1H, J = 1.9, 1.9 Hz, H-2), 3.82 (dd, 1H, J = 9.4, 9.4 Hz, H-4'), 3.80-3.72 (m, 2H, H-5, H-6a), 3.71 (m, 4H, H-6b, H-6a', H-6b', H-A1), 3.54 (ddd, 1H, J = 6.4, 6.4, 9.6 Hz, H-A2), 2.50-2.00 (m, 2H, H-C1, H-C2), 1.66-1.54 (m, 2H, H-B1, H-B2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 138.61, 138.41, 138.36, 138.33, 138.15 ($\underline{C}_6H_5CH_2O$), 138.03, 137.93 ($\underline{C}_6H_5CH_2O$), 128.47, 128.44, 128.32, 128.27, 128.23, 127.87, 127.81, 127.78, 127.73, 127.67, 127.54, 127.49, 127.45 ($\underline{C}_6H_5CH_2O$ and C-D), 114.85 (C-E), 101.29 (C-1', $^1J_{C-H}$ = 170.3 Hz), 97.42 (C-1, $^1J_{C-H}$ = 167.2 Hz), 80.02 (C-3'), 78.64 (C-3), 77.71 (C-2), 75.29 (C-4), 74.97, 74.83 ($O\underline{C}H_2Ph$), 74.42 (C-4'), 73.47, 73.39, 72.16, 72.03 ($O\underline{C}H_2Ph$), 71.89, 69.16 (C-6 and C-6'), 68.73 (C-2'), 67.11 (C-A), 30.28 (C-C), 28.61 (C-B); ESI HRMS (*m/z*): Calcd for $C_{59}H_{66}O_{11}Na$ 973.4497, found 973.4498.

5-(2-Aminoethylthio)pentenyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside-(1→3)-2,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**80**).

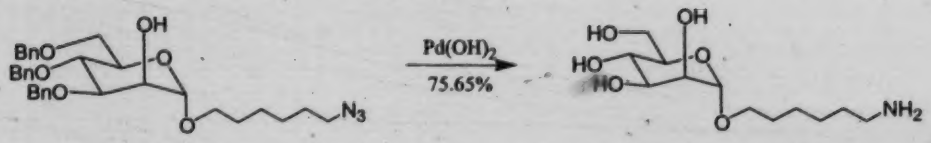


79 80

Disaccharide **79**, dissolved in freshly distilled CH_2Cl_2 (1mL) was placed in a quartz reaction tube and vigorously mixed with dry MeOH (5mL) until a mono-phasic layer was obtained. 2-Aminoethanethiol hydrochloride (0.7919g, 6.97mmol) was added and the mixture was stirred until a clear solution was obtained. Argon gas was bubbled through the solution for 2 min. The reaction was continuously stirred and irradiated with long wave (365nm) UV light overnight. TLC in 4:1 toluene-ethyl acetate revealed the absence of starting materials and TLC in 10:1 dichloromethane-methanol indicated the presence of product. The reaction mixture was diluted with CH_2Cl_2 and successively washed with saturated aqueous $NaHCO_3$, H_2O, and saturated aqueous brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. Flash silica gel column chromatography of the residue using 20:1:0.2 CH_2Cl_2-MeOH-Et_3N to 10:1:0.2 CH_2Cl_2-MeOH-Et_3N stepwise as eluants afforded compound **80** (0.084g, 80%) as a syrup; $[\alpha]_D^{25}$ +44.2 (*c* 1.03, CH_3OH); ^1H NMR (500 MHz, $CDCl_3$): δ_H, 7.36-7.13 (m, 30 H, aromatic), 5.05 (d, 1H, J = 1.7 Hz, H-1'), 4.83 (s, 1H, H-1), 4.82 (d, 1H, ^2J = 11.2 Hz, OCH_2Ph), 4.67 (d,

1H, ^2J = 12.0 Hz, OCH_2Ph), 4.61(s, 2H, OCH_2Ph), 4.60 (d, 2H, ^2J = 11.7 Hz, OCH_2Ph), 4.57 (d, 1H, ^2J = 12.0 Hz, OCH_2Ph), 4.49 (d, 2H, ^2J = 12.0 Hz, OCH_2Ph), 4.48 (d, 1H, ^2J = 10.9 Hz, OCH_2Ph), 4.86 (d, 1H, ^2J = 10.5 Hz, OCH_2Ph), 4.44 (d, 1H, ^2J = 11.8 Hz, OCH_2Ph), 4.02 (dd, 1H, J = 3.0, 9.5 Hz, H-3), 3.95 (dd, 1H, J = 4.9, 4.9 Hz, H-2'), 3.90 (dd, 1H, J = 9.4, 9.4 Hz, H-5'), 3.86 (dd, 1H, J = 1.9, 1.9 Hz, H-2), 3.84 (dd, 1H, J = 3.0, 9.2 Hz, H-3'), 3.74 (dd, 1H, J = 9.6, 9.6 Hz, H-4'), 3.72-3.61(m, 5H, H-5, H-6a, H-6b, H-6a', H-A1), 3.59 (dd, 1H, J = 5.0, 5.7 Hz, H-6b'), 3.36 (ddd, 1H, J = 5.9, 5.9, 9.6 Hz, H-A2), 3.01 (t, 1H, J = 6.8 Hz, H-H1, H-H2), 2.71 (t, 1H, J = 6.7 Hz, H-G1, H-G2), 5.51 (t, 1H, J = 7.3 Hz, H-E1, H-E2), 1.63-1.48 (m, 4H, H-B1, H-B2, H-D1, H-D2), 1.47-1.38 (m, 2H, H-C1, H-C2); ^{13}C NMR (125 MHz, CD_3OD): δ_C, 139.97, 139.74, 139.64, 139.62, 139.60, 139.56 ($\underline{C}_6H_5CH_2O$), 129.47, 129.46, 129.38, 129.30, 129.29, 129.26, 129.09, 129.05, 128.92, 128.84, 128.74, 128.69, 128.68, 128.64, 128.62 ($\underline{C}_6H_5CH_2O$),103.83 (C-1', $^1J_{C\text{-}H}$ = 171.2 Hz), 98.65 (C-1, $^1J_{C\text{-}H}$ = 168.2 Hz), 80.59 (C-3'), 80.13 (C-3), 79.07 (C-2), 76.31 (C-5'), 76.05, 75.85 ($O\underline{C}H_2Ph$), 75.81 (2C, C-1 and C-4'), 74.43, 74.35 ($O\underline{C}H_2Ph$), 73.49 (C-4), 73.43 (C-5), 73.26, 75.54 ($O\underline{C}H_2Ph$), 70.80 (C-6'), 70.26 (C-6), 69.40 (C-2'), 68.73 (C-A), 41.35 (C-H), 34.72 (C-G), 32.45 (C-E), 30.52, 30.04 (C-B and C-D), 26.54 (C-C); ESI HRMS (*m/z*): Calcd for $C_{61}H_{74}NO_{11}S$ 1028.4977, found 1028.4979.

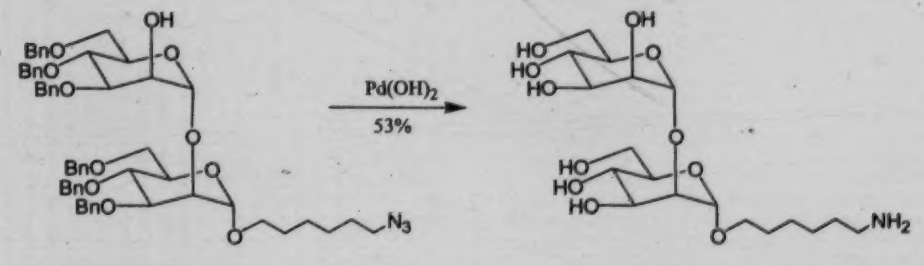
6'-Aminohexyl α-D-mannopyranoside (**81**).



72 **81**

A catalytic amount of $Pd(OH)_2$ (Pearlman's catalyst) was added to a solution of the tribenzylated mannopyranoside **72** (91mg, 0.16mmol) in dry MeOH (1.5mL). The atmosphere was flushed and saturated with H_2 gas (1 atm) and the mixture stirred overnight at room temperature. The reaction mixture was filtered with a 0.45 μm syringe filter and concentrated in vacuo. Purification of the residue on a Sep-Pak reverse phase C_{18} column using 1% AcOH in H_2O as the eluant afforded compound **81** (33.4mg, 76%); $[\alpha]_D^{25}$ +55.9 (*c* 1.50, CH_3OH); ^1H NMR (500 MHz, D_2O): δ_H, 4.86 (d, 1H, J = 1.3 Hz, H-1), 3.92 (dd, 1H, J = 1.7, 3.4 Hz, H-2), 3.88 (dd, 1H, J = 2.0, 12.4 Hz, H-6a), 3.80-3.69 (m, 3H, H-3, H-6b, H-A1), 3.64 (dd, 1H, J = 10.0, 10.0 Hz, H-4), 3.63 (m, 1H, H-5), 3.55 (ddd, 1H, J = 6.2, 6.2, 10.0 Hz, H-A2), 2.99 (t, 2H, J = 7.5 Hz, H-F1, H-F2), 1.72-1.57 (m, 4H, H-C1, H-C2, H-E1, H-E2), 1.47-1.34 (m, 4H, H-B1, H-B2, H-D1, H-D2); ^{13}C NMR (125 MHz, D_2O): δ_C, 100.53 (C-1, $^1J_{C\text{-}H}$ = 171.1 Hz), 73.63 (C-5), 71.53 (C-3), 70.95 (C-2), 68.57 (C-A), 67.66 (C-4), 61.83 (C-6), 40.32 (C-F), 29.12, 27.49 (C-B and C-E), 26.22, 25.71 (C-C and C-D); ESI HRMS (*m/z*): Calcd for $C_{12}H_{26}NO_6$ 280.1755, found 280.1756.

6'-Aminohexyl α-D-mannopyranosyl-(1→2)-α-D-mannopyranoside (**82**).

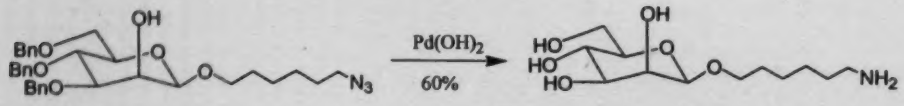


74 **82**

Trisaccharide **74** (43mg, 0.043mmol) was dissolved in MeOH (1.5mL). A catalytic amount of palladium (II) hydroxide was added and the solution stirred under H_2 (1atm) for 24 h. The reaction mixture was filtered through a 0.22μm syringe filter and purified on a reverse phase C_{18} Sep-Pak column eluting with 1% aqueous AcOH. Disaccharide **82** was obtained as a white solid after lyophilization (10mg, 53%); $[\alpha]_D^{25}$ +40.3 (*c* 0.37, CH_3OH); ^1H NMR (600 MHz, D_2O): δ_H, 5.09 (d, 1H, *J* = 1.1 Hz, H-1), 5.02 (d, 1H, *J* = 1.2 Hz, H-1'), 4.07 (dd, 1H, *J* = 2.0, 3.3 Hz, H-2'), 3.95 (dd, 1H, *J* = 1.8, 3.2 Hz, H-2), 3.91-3.86 (m, 3H, H-3, H-6a, H-6a'), 3.44 (dd, 1H, *J* = 3.4, 9.7 Hz, H-3'),3.79-3.69 (m, 4H, H-5', H-6b, H-6b', H-A1), 3.68 (dd, 1H, *J* = 9.8, 9.8 Hz, H-4), 3.64-3.58 (m, 2H, H-4', H-5), 3.54 (ddd, 1H, *J* = 6.0, 6.0, 10.0 Hz, H-A2), 2.99 (t, 2H, *J* = 7.5 Hz, H-F1, H-F2), 1.72-1.54 (m, 4H, H-C1, H-C2, H-E1, H-E2), 1.46-1.34 (m, 4H, H-B1, H-B2, H-D1, H-D2); ^{13}C NMR (125 MHz, D_2O): δ_C, 103.24 (C-1, $^1J_{C\text{-}H}$ = 172.2 Hz), 98.94 (C-1', $^1J_{C\text{-}H}$ = 172.2 Hz), 79.63 (C-2), 74.17 (C-5'), 73.64 (C- 5), 71.18 (C-3 and C-3'), 70.81 (C-2'), 68.65 (C-A), 67.85 (C-4), 67.79 (C-4'), 62.03, 61.83 (C-6 and C-6'),

40.32 (C-F), 29.07, 27.49 (C-B and C-E), 26.21, 25.74 (C-C and C-D); ESI HRMS (*m/z*): Calcd for $C_{18}H_{36}NO_{11}$ 442.2283, found 442.2280.

6'-Aminohexyl β-D-mannopyranoside (**83**).

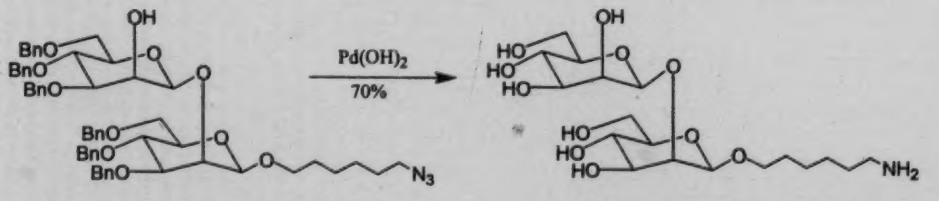


65 83

A catalytic amount of $Pd(OH)_2$ (Pearlman's catalyst) was added to a solution of compound **65** (30mg, 0.052mmol) in dry MeOH (5mL) and AcOH (2 drops). The atmosphere was flushed and saturated with H_2 gas (1 atm). The mixture was stirred overnight at room temperature after which additional AcOH (1 drop) was added and the atmosphere again flushed with H_2 gas and the reaction mixture stirred for 24 more h. The reaction mixture was filtered with a 0.45μm syringe filter and concentrated *in vacuo*. Purification of the residue on a Sep-Pak reverse phase C_{18} column eluting with 1% AcOH in H_2O as eluant afforded compound **83** (8.7mg, 60%); $[\alpha]_D^{25}$ -36.1 (*c* 0.62, CH_3OH); ^1H NMR (600 MHz, CD_3OD): δ_H, 4.48 (d, 1H, $J \leq 1$ Hz, H-1), 3.90 (ddd, 1H, J = 6.5, 6.5, 9.5 Hz, H-A1), 3.85 (dd, 1H, J = 2.3, 11.7 Hz, H-6a), 3.83 (dd, 1H, $J \leq 1, \approx 3.3$ Hz, H-2), 3.72 (high order dd, 1H, H-6b), 3.56 (ddd, 1H, J = 6.4, 6.4, 9.7 Hz, H-A2), 3.55 (dd, 1H, J = 9.6, 9.6 Hz, H-4), 3.43 (high order dd, 1H, H-3), 3.18 (ddd, 1H, J = 2.4, 5.5, 9.6 Hz, H-5), 2.90 (t, 2H, J = 7.4 Hz, H-F1, H-F2), 1.92 (s, 3H, OAc counter ion), 1.72-1.58 (m, 4H, H-C1, H-C2, H-E1, H-E2), 1.51-1.37 (m, 4H, H-B1, H-B2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 100.82 (C-1, $^1J_{C\text{-}H}$ = 156.0 Hz), 78.26 (C-5), 75.34 (C-3),

72.61 (C-2), 70.37 (C-A), 68.49 (C-4), 62.70 (C-6), 40.58 (C-F), 30.23, 28.38 (C-B and C-E), 26.94, 26.58 (C-C and C-D); ESI HRMS (*m/z*): Calcd for $C_{12}H_{26}NO_6$ 280.1755, found 280.1755.

6'-Aminohexyl β-D-mannopyranosyl-(1→2)-β-D-mannopyranoside (**84**).

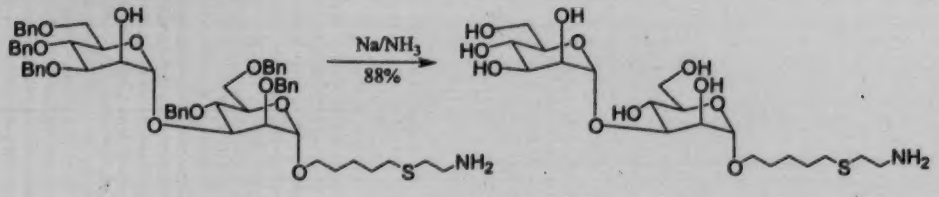


69 **84**

Disaccharide **69** (20mg, 0.02mmol) was dissolved in MeOH (2mL) and AcOH (0.5mL). A catalytic amount of Palladium (II) hydroxide was added and the solution shaken under H_2 (60 p.s.i) for 2 days. The reaction mixture was filtered through a 0.22μm membrane filter and purified on a C_{18} reverse phase Sep-Pak column eluting with 1% aqueous AcOH. The deprotected disaccharide **84** was obtained as a white solid after lyophilization (6.0mg, 71%); $[\alpha]_D^{25}$ -37.9 (*c* 0.42, CH_3OH); ^1H NMR (600 MHz, CD_3OD): δ_H, 4.77 (d, 1H, $J \leq 1$ Hz, H-1'), 4.57 (d, 1H, $J \leq 1$ Hz, H-1), 4.10 (d, 1H, $J = 3.3$ Hz, H-2), 3.97 (d, 1H, $J = 3.2$ Hz, H-2'), 3.93 (ddd, 1H, $J = 6.4, 6.4, 9.5$ Hz, H-A1), 3.88 (dd, 1H, $J = 2.3, 11.8$ Hz, H-6a), 3.86 (dd, 1H, $J = 2.3, 11.9$ Hz, H-6a'), 3.71 (dd, 1H, $J = 5.9, 11.8$ Hz, H-6b), 3.67 (dd, 1H, $J = 6.6, 12.0$ Hz, H-6b'), 3.56 (ddd, 1H, $J = 6.0, 6.0, 9.6$ Hz, H-A2), 3.53 (dd, 1H, $J = 9.6, 9.6$ Hz, H-4), 3.52 (dd, 1H, $J = 9.6, 9.6$ Hz, H-4'), 3.46 (high order dd, 1H, H-3), 3.35 (high order dd, 1H, H-3), 3.20 (ddd, 1H, $J = 2.3, 5.8, 9.3$ Hz, H-5), 3.15 (m,

1H, H-5), 2.93 (t, 2H, J = 7.3 Hz, H-F1, H-F2), 1.97 (S, 3H, OAc counter ion), 1.72-1.59 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.50-1.39 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, CD_3OD): δ_C, 102.29 (C-1', $^1J_{C\text{-}H}$ = 160.6 Hz), 102.05 (C-1, $^1J_{C\text{-}H}$ = 156.0 Hz), 79.25 (C-2), 78.75 (C-5'), 78.51 (C-5), 75.42 (C-3'), 74.63 (C-3), 72.08 (C-2'), 70.39 (C-A), 69.00, 68.56 (C-4 and C-4'), 62.98 (C-6'), 62.65 (C-6), 40.74 (C-F), 30.45, 28.70 (C-B and C-E), 27.06, 26.88 (C-C and C-D); HRMS (*m/z*): Calcd for $C_{18}H_{36}NO_{11}$ 442.2283, found 442.2282.

5-(2-Aminoethylthio)pentenyl α-D-mannopyranoside-(1→3)-α-D-mannopyranoside (**85**).

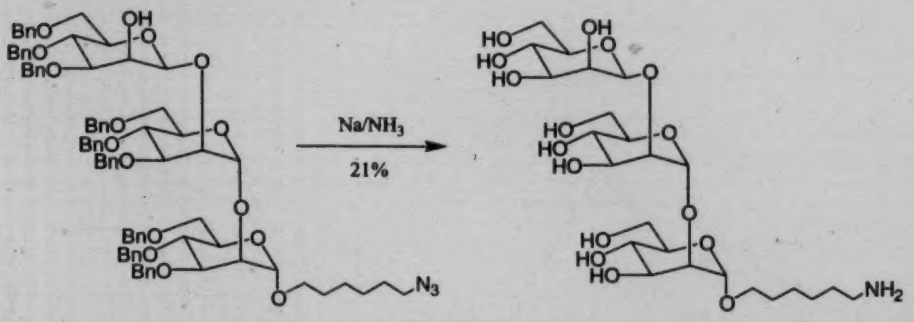


80 **85**

Compound **80** (59mg, 0.058mmol) was dissolved in dry THF (2mL) and MeOH (80μL) and transferred to a 3-neck flask containing liquid NH_3 and sodium metal at –78 °C. The dark blue reaction solution was stirred vigorously but started to lighten in color after 30 min. Another piece of sodium was added and after 1 h, methanol was added until a neutralized milky white solution was obtained. Argon was streamed into the vessel until the solvent had evaporated and the residue dissolved in water. The resulting aqueous solution was chromatographed on a reversed-phase Hamilton HPLC column and a gradient of H_2O-CH_3OH (0→ 80%) was employed as eluant. Compound

85 was lyophilized and obtained as a white solid (25mg, 88%); $[\alpha]_D^{25}$ +71.3 (*c* 0.38, CH_3OH); ^1H NMR (500 MHz, CD_3OD): δ_H, 5.06 (d, 1H, *J* = 1.7 Hz, H-1'), 4.71 (d, 1H, *J* = 1.8 Hz, H-1), 4.01 (dd, 1H, *J* = 1.9, 3.2 Hz, H-2), 3.96 (dd, 1H, *J* = 1.7, 3.3 Hz, H-2'), 3.83 (dd, 1H, *J* = 2.3, 6.5 Hz, H-6a), 3.81 (dd, 1H, *J* = 2.3, 4.3 Hz, H-3'), 3.80-3.65 (m, 7H, H-4, H-6b, H-3, H-5', H-6a', H-6b', H-A1), 3.61 (dd, 1H, *J* = 9.5, 9.5 Hz, H-4'), 3.55 (ddd, 1H, *J* = 2.4, 5.9, 9.5 Hz, H-5), 3.42 (ddd, 1H, *J* = 3.2, 3.2, 6.1 Hz, H-A2), 2.77 (broad t, 2H, *J* = 6.5 Hz, H-H1, H-H2), 2.61 (t, 2H, *J* = 6.6 Hz, H-G1, H-G2), 2.53 (t, 2H, *J* = 7.3 Hz, H-E1, H-E2), 1.66-1.56 (m, 4H, H-B1, H-B2, H-D1, H-D2), 1.54-1.44 (m, 2H, H-C1, H-C2); ^{13}C NMR (125 MHz, CD_3OD): δ_C,103.94 (C-1', $^1J_{C-H}$ = 169.9 Hz), 101.67 (C-1, $^1J_{C-H}$ = 168.7 Hz), 80.74 (C-3), 74.89 (C-5 and C-5'), 72.51 (C-3'), 72.14 (C-2'), 71.57 (C-2), 68.79 (C-4'), 68.51 (C-A), 67.67 (C-4), 62.89 (C-6'), 62.84 (C-6), 41.74 (C-H), 35.87 (C-G), 32.47 (C-E), 30.73, 30.15 (C-B and C-D), 26.55 (C-C); ESI HRMS (*m/z*): Calcd for $C_{19}H_{38}NO_{11}S$ 488.2160, found 488.2159.

6'-Aminohexyl β-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→2)-α-D-mannopyranoside (**86**).

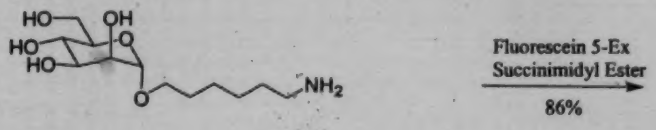


76 **86**

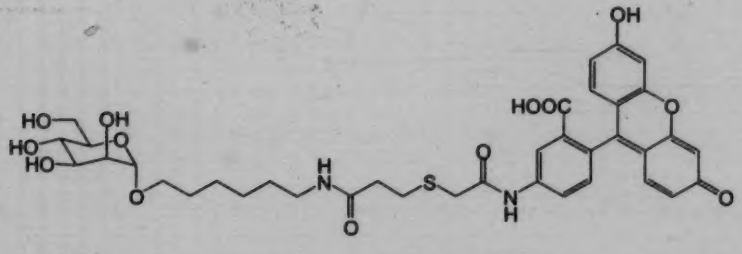
Benzylated trisaccharide **76** (25.0mg, 0.017mmol) was dissolved in dry THF (2mL) and MeOH (40μL) and transferred to a 3-neck flask containing sodium metal in liquid NH_3 at –78 °C. The dark blue reaction solution was stirred vigorously. When the colour of the mixture started to lighten in color after 30 min another piece of sodium was added. This procedure was repeated after another 30 min. After 1.5 h, methanol was added until a milky white solution was obtained. Argon was streamed into the vessel until the solvent had evaporated and the residue dissolved in water. The resulting aqueous solution was chromatographed on a Hamilton HPLC column using a gradient of H_2O-CH_3OH (0→ 80%) as the eluant. Trisaccharide **86** was lyophilized and obtained as a white solid (2.2mg, 21%); $[\alpha]_D^{25}$ +20.0 (*c* 0.13, CH_3OH); ^1H NMR (600 MHz, $CDCl_3$): δ_H, 5.06 (d, 1H, J = 1.7 Hz, H-1'), 5.04 (d, 1H, J = 1.8 Hz, H-1), 4.66 (d, 1H, J = 0.67 Hz, H-1''), 4.21 (dd, 1H, J = 1.8, 3.3 Hz, H-2'), 3.89-3.80 (m, 5H, H-2, H-2'', H-6a, H-6a', H-6a''), 3.77 (dd, 1H, J = 3.3, 9.3 Hz, H-3), 3.75-3.62 (m, 6H, H-3', H-5, H-6b, H-6b', H-6b'', H-A1), 3.58-3.51 (m, 3H, H-4, H-4', H-4''), 3.49 (m, 1H, H-5'), 3.47-3.41 (m, 2H, H-

3'', H-A2), 3.23 (m, 1H, H-5''), 2.91 (t, 2H, J = 7.5 Hz, C-F1, C-F2), 1.69-1.53 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.48-1.35 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C, 101.73 (C-1', $^1J_{C-H}$ = 168.9 Hz), 99.91 (C-1, $^1J_{C-H}$ = 168.9 Hz), 99.87 (C-1'', $^1J_{C-H}$ = 157.5 Hz), 80.78 (C-5), 80.63 (C-2), 78.61 (C-5''), 78.26 (C-2'), 75.21 (C-3''), 74.77 (C-5'), 72.62 (C-2''), 72.26 (C-3), 71.88 (C-3'), 69.19, 69.16, 68.51 (C-4, C-4' and C-4''), 68.30 (C-A), 63.16, 62.98, 62.94 (C-6, C-6', and C-6''), 58.86 (C-F), 30.26, 28.84 (C-B and C-E), 27.17, 26.82 (C-C, C-D); ESI HRMS (*m/z*): Calcd for $C_{24}H_{46}NO_{16}$ 604.2811, found 604.2811.

14-*N*-[3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]-7,14-di-aza-8,13-di-oxo-11-thia-tetradecyl α-D-mannopyranoside (**87**).



81

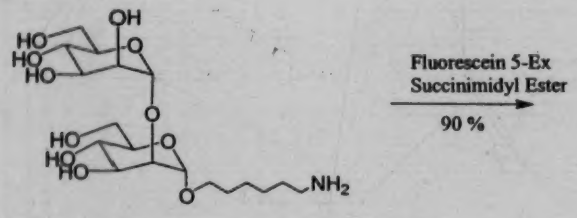


87

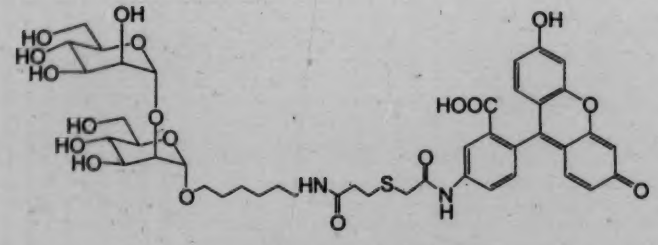
To a solution of **81** (1.5mg, 54mmol) in MeOH (50μL) was added H_2O (40μL), Et_3N (2μL), and fluorescein-5-EX succinimidyl ester (2.0mg, 0.0034mmol) in CH_3CN (50μL). The reaction mixture was vortexed and centrifuged. After 2 h the reaction mixture was diluted with water to a volume

of 1mL. The reaction product was purified by HPLC on a reverse phase C_{18} column using a gradient of H_2O-CH_3CN (0→ 80%) as eluant. Labelled monosaccharide **87** was obtained as a bright orange solid after lyophilization (2.2mg, 86%); ^{1}H NMR (500 MHz, D_2O): δ_H, 7.95 (d, 1H, J = 2.1 Hz, aromatic), 7.79 (m, 1H, aromatic), 7.29-7.17 (m, 3H, aromatic), 6.76-6.68 (m, 4H, aromatic), 4.75 (overlap with HDO, H-1), 3.84 (dd, 1H, J= 1.8, 3.4 Hz, H-2), 3.80 (broad dd, 1H, $J \leq 1$, $J \approx 11.7$ Hz, H-6a), 3.74-3.68 (m, 2H, H-3, H-6b), 3.61 (dd, 1H, J = 9.8, 9.8 Hz, H-4), 3.58 (ddd, J = 7.0, 7.0, 9.7 Hz, H-A1), 3.53 (m, 1H, H-5), 3.52 (s, 2H, H-L1. H-L2), 3.38 (ddd, 1H, J = 6.2, 6.2, 9.8 Hz, H-A2), 3.13 (t, 2H, J = 6.9 Hz, H-F1, H-F2), 2.97 (t, 2H, J = 6.7 Hz, H-J1, H-J2), 2.62 (t, 2H, 6.8 Hz, H-I1, H-I2), 1.54-1.38 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.37-1.10 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, CD_3OD): δ_C, 173.83 (2C, C=O), 172.61 (C=O), 171.39 (C=O), 170.46 (aromatic), 157.97-157.84 (aromatic), 156.52 (aromatic), 132.02, (aromatic), 129.64 (aromatic), 123.63 (aromatic), 114.43 (aromatic), 103.90 (aromatic), 101.76 (C-1, $^1J_{C\text{-}H}$ = 156.1 Hz), 78.26 (C-5), 75.37 (C-3), 72.60 (C-2), 70.49 (C-A), 68.63 (C-4), 62.91 (C-6), 40.40 (C-F), 37.22 (C-L), 36.96 (C-I), 30.61 (C-J), 30.32, 29.68 (C-B and C-E), 27.74, 26.79 (C-C and C-D); ESI HRMS (negative mode) (*m/z*): Calcd for $C_{37}H_{41}N_2O_{13}S$ 753.2335, found 753.2332 $[MH]^-$.

14-*N*-[3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]-7,14-di-aza-8,13-di-oxo-11-thia-tetradecyl α-D-mannopyranosyl-(1→2)-α-D-mannopyranoside (**88**).



82

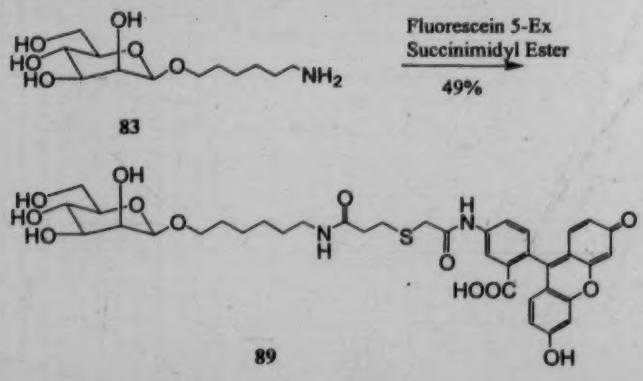


88

To a solution of **82** (3.0mg, 6.8mmol) in MeOH (40μL) was added H_2O (40 μL), Et_3N (4μL), and fluorescein-5-EX succinimidyl ester (5.3mg, 0.0090mmol) in CH_3CN (132.5μL). The reaction mixture was vortexed and centrifuged. After 1 h the reaction mixture was diluted with water to a volume of 1mL. The reaction product was purified by HPLC on a reverse phase C_{18} column using a gradient of H_2O-CH_3CN (0→ 80%) as eluant. Labelled disaccharide **88** was obtained as a bright orange solid after lyophilization (5.6mg, 90%). ^1H NMR (600 MHz, D_2O): δ_H, 7.98 (d, 1H, $J \leq 1$, aromatic), 7.72 (m, 1H, aromatic), 7.15-7.05 (m, 3H, aromatic), 6.76-6.64 (m, 4H, aromatic), 5.00 (d, 1H, J = 1.1 Hz, H-1), 4.96 (d, 1H, J = 1.2 Hz, H-1'), 4.04 (dd, 1H, J = 1.8, 3.3 Hz, H-2'), 3.86-3.78 (m, 5H, H-2, H-3, H-3', H-6a, H-6a'), 3.75-3.67 (m, 3H, H-5', H-6b, H-6b'), 3.65 (dd, 1H, J = 9.8, 9.8 Hz, H-

4), 3.61 (dd, 1H, J = 9.7, 9.7 Hz, H-4'), 3.58 (ddd, 1H, J = 7.0, 7.0, 9.6 Hz, H-A1), 3.52 (ddd, 1H, J = 2.0, 5.6, 9.7 Hz, H-5), 3.48 (m, 2H, H-L1, H-L2), 3.39 (ddd, 1H, J = 6.2, 6.2, 10.1 Hz, H-A2), 3.11 (m, 2H, J = 7.5 Hz, H-F1, H-F2), 2.94 (m, 2H, H-J1, H-J2), 2.58 (m, 2H, H-I1, H-I2), 1.54-1.34 (m, 4H, H-C1, H-C2, H-E1, H-E2), 1.31-1.14 (m, 4H, H-B1, H-B2, H-D1, H-D2); ^{13}C NMR (125 MHz, D_2O): δ_C, 174.70 (2C, C=O), 172.27 (C=O), 158.38 (C=O), 139.52(aromatic), 132.47, (aromatic), 122.97-121.58 (aromatic), 115.67 (aromatic), 104.17 (aromatic), 103.25 (C-1', $^1J_{C\text{-}H}$ = 171.2 Hz), 98.94 (C-1, $^1J_{C\text{-}H}$ = 171.4 Hz), 79.60 (C-2), 74.10 (C-5'), 73.55 (C-5), 71.20, 71.18, 70.83 (C-2', C-3, and C-3'), 68.66 (C-A), 67.80, 67.75 (C-4 an C-4'), 61.94, 61.71 (C-6 and C-6'), 40.20 (C-F), 37.12 (C-L), 36.35 (C-I), 29.44 (C-J), 29.31, 29.06 (C-B and C-E), 26.661, 25.89 (C-C and C-D); ESI HRMS (negative mode) (m/z): Calcd for $C_{43}H_{51}N_2O_{18}S$ 915.2863, found 915.2858 $[MH]^-$.

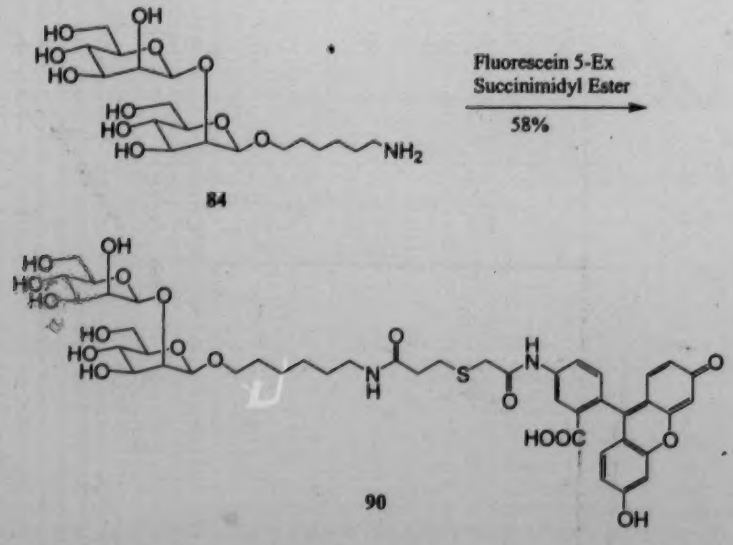
14-*N*-[3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]-7,14-di-aza-8,13-di-oxo-11-thia-tetradecyl β-D-mannopyranoside (**89**).



To a solution of **83** (2mg, 0.007mmol) in MeOH (51μL) was added water (40μL), Et_3N (4μL), and fluorescein-5-EX succinimidyl ester (5.07mg) in CH_3CN (125μL). The reaction mixture was vortexed and centrifuged. After 2 h the reaction mixture was diluted with water to a volume of 2mL. The reaction product was purified by HPLC on a reverse phase C_{18} column using a gradient of H_2O-CH_3CN (0→ 80%) as the eluant. Labelled monosaccharide **89** was obtained as a bright orange solid after lyophilization (2.4mg, 49%); ^1H NMR (600 MHz, CD_3OD): δ_H, 8.20 (d, 1H, J = 2.2 Hz, aromatic), 7.95 (dd, 1H, J = 2.3, 8.4 Hz, aromatic), 7.18 (d, 1H, J = 8.2 Hz, aromatic), 7.05-6.99 (m, 2H, aromatic), 6.64 (d, 2H, J = 2.3 Hz, aromatic), 6.61 (d, 1H, J = 2.2 Hz, aromatic), 6.60 (d, 1H, J = 2.3 Hz, aromatic), 4.47 (d, 1H, J = 0.88 Hz, H-1), 3.88 (ddd, 1H, J = 6.6, 6.6, 9.6 Hz, H-A1), 3.85 (dd, 1H, J = 2.4, 11.8 Hz, H-6a), 3.82 (dd, 1H, $J \leq 1, \approx 3.3$ Hz, H-2), 3.70 (dd, 1H, J = 5.8, 11.9 Hz, H-6b), 3.54 (dd, 1H, J = 9.6, 9.6 Hz, H-4), 3.51 (ddd, 1H, J = 6.6, 6.6, 9.6 Hz, H-A2), 3.42 (dd, 1H, J = 1.5, 5.6 Hz, H-3), 3.41 (s, 2H, H-L1, H-L2), 3.20-3.17(m, 3H, H-5, H-F1 and H-F2), 2.96 (t, 2H, H-J1, H-J2), 2.56 (t, 2H, 7.0 Hz, H-I1,

H-I2), 1.64-1.46 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.44-1.28 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, CD_3OD): δ_C, 172.66 (2C, C=O), 170.02 (C=O), 131.37, (aromatic), 113.68 (aromatic), 102.88 (aromatic), 100.35 (C-1', $^1J_{C\text{-}H}$ = 171.1 Hz), 73.41 (C-5), 71.44 (C-3), 71.08 (C-2), 67.49 (C-A), 67.26 (C-4), 61.76 (C-6), 39.20 (C-F), 36.03 (C-L), 35.79 (C-I), 29.29 (C-J), 29.11, 28.50 (C-B and C-E), 26.53, 25.81 (C-C and C-D); ESI HRMS (negative mode) (*m/z*): Calcd for $C_{37}H_{41}N_2O_{13}S$ 753.2335; found 753.2335 $[MH]^-$.

14-*N*-[3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]-7,14-di-aza-8,13-di-oxo-11-thia-tetradecyl β-D-mannopyranosyl-(1→2)-β-D-mannopyranoside (**90**).

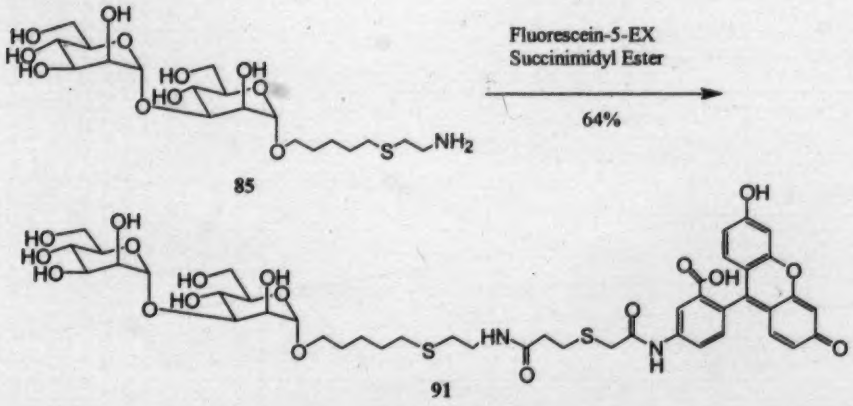


To a solution of disaccharide **84** (3.0mg, 0.0067mmol) in MeOH (100μL) was added H_2O (40μL), Et_3N (3.92μL), and fluorescein-5-EX succinimidyl ester (5.4mg, 0.0091mmol) in CH_3CN (135μL). The reaction mixture was vortexed and centrifuged. After 1 h the reaction mixture was diluted with water to a

volume of 1mL. The reaction product was purified by HPLC on a reverse phase C_{18} column using a gradient of H_2O-CH_3CN (0→ 80%) as the eluant. Labelled disaccharide **90** was obtained as a bright orange solid after lyophilization (3.6mg, 58%); ^{1}H NMR (600 MHz, CD_3OD): δ_H, 8.12 (d, 1H, J = 2.2 Hz, aromatic), 7.97 (dd, 1H, J = 2.2, 8.2 Hz, aromatic), 7.18 (d, 1H, J = 8.2 Hz, aromatic), 7.12 (d, 1H, J = 8.9 Hz, aromatic), 6.60 (d, 1H, J = 2.2 Hz, aromatic), 6.58 (m, 3H, aromatic), 4.77 (d, 1H, $J \leq 1$ Hz, H-1'), 4.54 (d, 1H, $J \leq 1$ Hz, H-1), 4.09 (d, 1H, J = 3.4 Hz, H-2), 3.98 (d, 1H, J = 3.2 Hz, H-2'), 3.91 (ddd, 1H, J = 6.4, 6.4, 9.5 Hz, H-A1), 3.87 (dd, 1H, J = 2.3, 5.1 Hz, H-6a'), 3.85 (dd, 1H, J = 2.4, 5.3 Hz, H-6a), 3.69 (dd, 1H, J = 6.0, 11.9 Hz, H-6b), 3.67 (dd, 1H, J = 6.4, 11.9 Hz, H-6b'), 3.52 (dd, 1H, J = 9.5, 9.5 Hz, H-4'), 3.51 (dd, 1H, J = 9.6, 9.6 Hz, H-4), 3.50 (ddd, 1H, J = 6.2, 6.2, 9.9 Hz, H-A2), 3.45 (dd, 1H, J = 3.4, 9.7 Hz, H-3), 3.42 (s, 2H, H-L1, H-L2), 3.39 (dd, 1H, J = 3.3, 9.5 Hz, H-3'), 3.21-3.14 (m, 4H, H-5, H-5', H-F1, H-F2), 2.96 (t, 2H, J = 7.1 Hz, H-J1, H-J2), 2.56 (t, 2H, J = 7.0 Hz, H-I1, H-I2), 1.62-1.47 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.42-1.32 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, CD_3OD): δ_C, 173.88 (2C, C=O), 171.15 (C=O), 159.34 (C=O), 140.76 (aromatic), 132.53, (aromatic), 130.99 (aromatic), 122.02 (aromatic), 121.52 (aromatic), 114.60 (aromatic), 104.12 (aromatic), 102.22 (C-1', $^1J_{C\text{-}H}$ = 161.0 Hz), 102.02 (C-1, $^1J_{C\text{-}H}$ = 156.1 Hz), 79.09 (C-2), 78.65, 78.46 (C-5 and C-5'), 75.30 (C-3'), 74.56 (C-3), 72.14 (C-2'), 70.50 (C-A), 69.10 (C-4), 68.58 (C-4'), 62.95 (C-6), 62.76 (C-6'), 40.41 (C-F), 37.28 (C-L), 37.06 (C-I), 30.71 (C-J), 30.39, 29.72 (C-B and C-E), 27.66,

27.01 (C-C and C-D); ESI HRMS (negative mode) (*m/z*): Calcd for $C_{43}H_{51}N_2O_{18}S$ 915.2863, found 915.2865 $[MH]^-$.

16-*N*-[3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]-9,16-di-aza-10,15-di-oxo-6,13-di-thia-tetradecyl α-D-mannopyranoside-(1→3)-α-D-mannopyranoside (**91**).



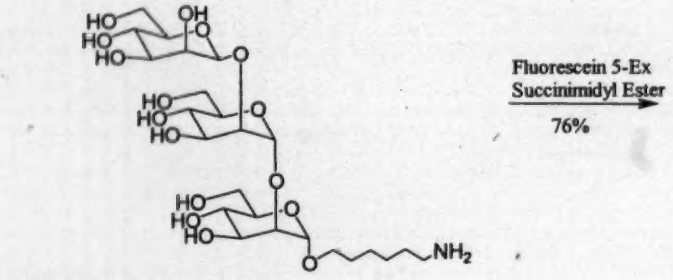
85

91

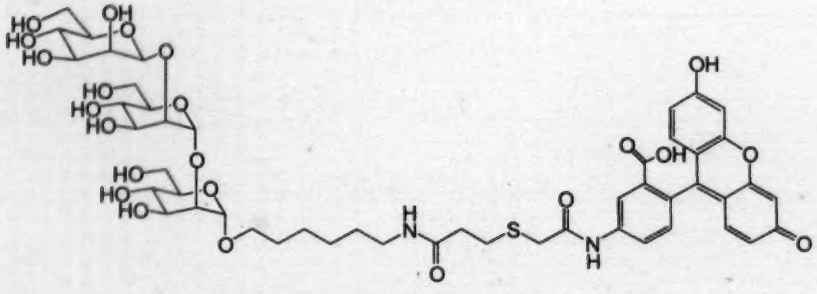
To a solution of 1,3-linked disaccharide **85** (4.5mg, 9.3μmol) in MeOH (73.5μL) was added H_2O (40μL), Et_3N (3.6μL), and fluorescein-5-EX succinimidyl ester (5.0mg) in CH_3CN (125μL). The reaction mixture was vortexed and centrifuged. After 1 h the reaction mixture was diluted with water to a volume of 1mL. The reaction product was purified by HPLC on a reverse phase C_{18} column using a gradient of H_2O-CH_3CN (0→ 80%) as eluant. Labelled disaccharide **91** was obtained as a bright orange solid after lyophilization (5.5mg, 64%); ^{1}H NMR (500 MHz, CD_3OD): δ_H, 8.26 (d, 1H, J = 1.7 Hz, aromatic), 7.92 (dd, 1H, J = 2.0, 8.3 Hz, aromatic), 7.15 (d, 1H, J = 8.3 Hz, aromatic), 6.86 (d, 2H, J = 8.9 Hz, aromatic), 6.67 (d, 2H, J = 2.3 Hz, aromatic), 6.60 (d, 1H, J = 2.4 Hz, aromatic), 6.58 (d, 1H, J = 2.3 Hz,

aromatic), 5.06 (d, 1H, J = 1.7 Hz, H-1'), 4.70 (d, 1H, J = 1.7 Hz, H-1), 4.01 (dd, 1H, J = 1.8, 3.1 Hz, H-2), 3.96 (dd, 1H, J = 1.7, 3.4 Hz, H-2'), 3.86-3.66 (m, 9H, H-3, H-3', H-4, H-5', H-6a, H-6a', H-6b, H-6b', H-A1), 3.61 (dd, 1H, J = 9.6, 9.6 Hz, H-4'), 3.55 (ddd, 1H, J = 2.3, 5.8, 9.3 Hz, H-5), 3.42 (s, 2H, H-N1, H-N2), 3.40 (ddd, 1H, J = 6.3, 6.3, 9.8 Hz, H-A2), 3.35 (t, 2H, J = 6.8 Hz, H-H1, H-H2), 2.96 (t, 2H, J = 7.1 Hz, H-L1, H-L2), 2.62 (t, 2H, J = 7.2 Hz, H-H1, H-H2), 2.57 (t, 2H, J = 7.1 Hz, H-K1, H-K2), 2.54 (t, 2H, J = 7.6 Hz, H-E1, H-E2), 1.67-1.54 (m, 4H, H-B1, H-B2, H-D1, H-D2), 1.52-1.41 (m, 2H, H-C1, H-C2); ^{13}C NMR (125 MHz, CD_3OD): δ_C, 174.00 (2C, C=O), 171.28 (C=O), 141.44 (C=O), 131.26 (aromatic), 103.92 (C-1', $^1J_{C\text{-}H}$ = 170.1 Hz), 103.72 (aromatic), 101.67 (C-1, $^1J_{C\text{-}H}$ = 168.4 Hz), 80.75 (C-3), 74.88 (C-5 and C-5'), 72.52 (C-3'), 72.16 (C-2'), 71.57 (C-2), 68.81 (C-4'), 68.51 (C-A), 67.68 (C-4), 62.92 (C-6'), 62.87 (C-6), 40.41 (C-H), 37.22 (C-N), 36.92 (C-K), 32.61 (C-E), 32.12 (C-G), 30.55 (C-L), 30.13, 29.50 (C-B and C-D), 26.53 (C-C); ESI HRMS (negative mode) (*m/z*): Calcd for $C_{44}H_{53}N_2O_{18}S_2$ 961.2744, found 961.2740 $[MH]^-$.

14-*N*-[3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]-7,14-di-aza-8,13-di-oxo-11-thia-tetradecyl β-D-mannopyranosyl-(1→2)-(α-D-mannopyranosyl)-(1→2)-α-D-mannopyranoside (**92**).



86



92

To a solution of trisaccharide **86** (2.2mg, 0.0037mmol) in MeOH (80μL) was added H_2O (20μL), Et_3N (2μL), and fluorescein-5-EX succinimidyl ester (2.8mg) in CH_3CN (70μL). The reaction mixture was vortexed and centrifuged. After 2 h the reaction was diluted with water to a volume of 1mL. The reaction product was purified by HPLC on a reverse phase C_{18} column using a H_2O-CH_3CN (0→ 80%) gradient. Compound **92** was obtained as a bright orange solid after lyophilization (3.0mg, 76%); ^{1}H NMR (600 MHz, CD_3OD): δ_H, 8.12 (d, 1H, J = 2.2 Hz, aromatic), 7.97 (dd, 1H, J = 2.2, 8.2 Hz, aromatic), 7.18 (d, 1H, J = 8.4 Hz, aromatic), 7.12 (d, 1H, J = 9.7 Hz, aromatic), 6.62 (d, 1H, J = 2.3 Hz, aromatic), 6.59 (m, 3H, aromatic), 5.05 (d,

1H, J = 1.6 Hz, H-1'), 5.02 (d, 1H, J = 1.7 Hz, H-1), 4.65 (d, 1H, $J \leq 1$ Hz, H-1''), 4.20 (dd, 1H, J = 1.8, 3.4 Hz, H-2'), 3.89-3.78 (m, 6H, H-2, H-2'', H-6a, H-6b, H-6a', H-6a''), 3.75-3.63 (m, 6H, H-3, H-3' H-5, H-6b', H-6b'', H-A1), 3.57 (t, 1H, J = 9.6 Hz, H-4'), 3.56 (t, 1H, J = 9.7 Hz, H-4), 3.52 (t, 1H, J = 9.4 Hz, H-4''), 3.49 (ddd, 1H, J = 2.4, 6.0, 9.8 Hz, H-5'), 3.44 (dd, 1H, J = 3.2, 9.3 Hz, H-3''), 3.43-3.39 (m, 3H, H-A2, H-L1, H-L2), 3.23 (ddd, 1H, J = 2.4, 6.4, 9.5 Hz, H-5''), 3.18 (t, 2H, J = 7.0 Hz, H-F1, H-F2), 2.96 (t, 2H, J = 7.1 Hz, H-J1, H-J2), 2.56 (t, 2H, J = 7.0 Hz, H-I1, H-I2), 1.62-1.46 (m, 4H, H-B1, H-B2, H-E1, H-E2), 1.42-1.32 (m, 4H, H-C1, H-C2, H-D1, H-D2); ^{13}C NMR (125 MHz, CD_3OD): δ_C, 172.65 (2C, C=O), 170.02 (C=O), 158.74 (C=O), 139.67 (aromatic), 131.33, (aromatic), 129.75 (aromatic), 121.15 (aromatic), 120.86 (aromatic), 113.53 (aromatic), 103.01 (aromatic), 102.89 (C-1', $^1J_{C-H}$ = 169.2 Hz), 100.48 (C-1, $^1J_{C-H}$ = 169.5 Hz), 98.69 (C-1'', $^1J_{C-H}$ = 157.4 Hz), 79.52 (C-2), 77.35 (C-2'), 77.10 (C-3''), 73.96 (C-2''), 73.50, 71.42, 71.02, 70.67, 68.00, 67.95, 67.31, 61.88, 61.73, 61.69 (C-6, C-6', and C-6''), 56.27, 39.22 (C-F), 36.05 (C-L), 35.79 (C-I), 29.29 (C-J), 29.10, 28.50 (C-B and C-E), 26.53, 25.81 (C-C and C-D); ESI HRMS (negative mode) (*m/z*): Calcd for $C_{49}H_{61}N_2O_{23}S$ 1077.3391, found 1077.3390 $[MH]^-$.

6.3 Fungal Extract Generation and General Biological Assay

6.3a Strain and Culture Conditions:

The *Candida albicans* serotype A CAI4 strain was used in all fungal extract assays. CAI4 *Candida albicans* from a glycerol stock were streaked on YPD plates. After overnight incubation at 30 °C, a single colony of the fungi was inoculated into YPD media. The culture was incubated overnight and the cells harvested by centrifugation. The cell pellet was subsequently frozen at -70 °C.

6.3b Fungal Extract preparation:

The CAI4 cells were resuspended in 50mM HEPES, pH 7.0, containing 10% glycerol and lysed. Low speed centrifugation (5000 rpm) followed by filtration of the supernatant allowed the removal of cellular debris. After ultracentrifugation at 45,000 rpm, the supernatant was recovered and put aside (fraction S), whereas the pellet was resuspended in 50mM HEPES, pH 7.0 (fraction P). Solubilization of fraction P with detergents was carried out as specified in the assays below. Typically, a 0.2% POE fungal extract was achieved by addition of POE and subsequent shaking on a rocker for 60 min at 4 °C. Further ultracentrifugation at 45,000 rpm and resuspension of the pellet in 50mM HEPES, pH 7.0 afforded the crude enzyme extract fraction POE-P.

6.3c Small Scale Fungal Extract Assays:

All commercial reagents were used as supplied. All substrates were quantified by measuring the A_{492} and using an extinction coefficient of 68,000 $M^{-1}cm^{-1}$. The required amount of substrate solution was transferred to a micro centrifuge tube and the solvent removed by brief heating at 70 °C. The reagents were added in the order buffer, divalent ions $MnCl_2$ and/or $MgCl_2$, DTT, H_2O, α-GDP-mannose, and the specified fungal extract. The reactions were incubated in heat blocks at 30 °C. Aliquots of the reaction mixture were taken at the specified time points for each assay and the reaction quenched by the addition of an equal amount of stop mix (10mM EDTA, 1% SDS, 50% MeCN). The reaction progress was monitored by TLC and CE.

6.3d Large Scale Fungal Extract Assays:

All commercial reagents were used as supplied. All substrates were quantified by measuring the A_{492} and using an extinction coefficient of 68,000 $M^{-1}cm^{-1}$. The required amount of substrate solution was transferred to a micro centrifuge tube and the solvent removed under reduced pressure using a Savant Speed Vac Sc110. The reagents were added in the order buffer, divalent ions $MnCl_2$ and/or $MgCl_2$, DTT, H_2O, α-GDP-mannose, and fungal extract fraction P. The reactions were supplemented with additional α-GDP-mannose over time when specified. The reactions were incubated in heat blocks at 30 °C. Aliquots of the reaction mixture were taken at specified time points and were quenched by the addition of an equal amount of stop mix

(10mM EDTA, 1% SDS, 50% MeCN). The reaction progress was monitored by TLC and CE. Termination of the reactions was achieved by 5X dilution with H_2O.

6.3e α- and β-Mannosidase Assays:

The α-mannosidase was obtained from New England BioLabs (USA), and the β-mannosidase from Dr. S. Withers at the University of British Columbia (Canada). Both of these mannosidases are exo-glycosidases. The reagents were added in order buffer, BSA, H_2O, and mannosidase. The reactions were incubated in heat blocks at 37 °C. Aliquots of the reaction mixture were taken at the specified time points for each assay and were quenched by the 3X addition of H_2O. The reaction progress was monitored by TLC. The remainder of the reaction mixture was terminate by 3X dilution with H_2O.

6.3f CaMNT1p Assays:

The crude CaMNT1p enzyme extract was obtained from Dr. W. Wakarchuk from the NRC (Canada). The reagents were added as before and heated at 30 °C. Aliquots of the reaction mixture were taken at the specified time points for each assay and the reaction quenched by the addition of an equal amount of stop mix (10mM EDTA, 1% SDS, 50% MeCN). The reaction progress was monitored by TLC and CE.

6.3g Chromatography:

Analytical thin layer chromatography (TLC) was performed on silica gel 60-F_{254} (Merck). TLC plates were developed in 7:2:1:0.1 and 4:2:1:0.1 EtOAC:MeOH:H_2O:AcOH as eluant. Compounds were visualized under UV light. Reverse phase C_{18} silica gel cartridges (C_{18} SepPak) were obtained from Waters Corp. (Milford, USA). Purification of the large scale assays was conducted with an AKTA Explorer 10 FPLC with a tunable Monitor UV-900 absorbance detector. Separations were achieved with Sephasil Peptide C_{18} 12μm PRC semi-preparative columns. Combinations of water and methanol containing 0.1% AcOH, 20mM NH_4OAc, pH 6.0 and MeCN, and 10mM NH_4OAc, pH 5.0 and MeCN were used as eluents (flow rate 1 mL min^{-1}).

6.3h Analytical:

CE was performed with a Beckman (fullerton, CA) P/ACE MDQ equipped with a Laser Induced Fluorescence (LIF) detection system using a Laser Module Argon Ion (λ excitation = 488nm, λ emission = 520nm. The capillary was of bare silica 50μm · 60.2cm, with the detector at 50cm. The capillary was conditioned before each run by washing with 0.2 NaOH for 2 min, water for 2 min, and Tris-borate-EDTA, pH 8.28, for 2 min. Samples were introduced by pressure injection at 0.5 psi for 5-10 followed by a 0.5 psi x 5 seconds water injection. The separation was performed at 30kV, 13.0 μA for TBE and 30kV, 18.0 μA for TBES. Peak integration was performed with the 32 Karat Software, version 5.0. ^1H NMR spectra were recorded on Varian

INOVA 600 and 800 MHz spectrometers employing a cryoprobe. First order ^{1}H NMR chemical shifts are referenced as before to internal standards of the residual protonated solvent peaks. Assignments were made with the aid of GCOSY, GHSQC, GHMBC, and GTOCSY experiments. MALDI ionization mass spectra were recorded on a Voyager-DE STR TOF mass spectrometer. Electrospray ionization mass spectra were recorded on a Micromass Zabspec TOF mass spectrometer. Spectra were acquired at the mass spectrometry laboratories of the NRC and this department.

6.4 Specific Biological Assays

Assay 1: General Fungal Extract Assay with Acceptors **87-92**.

This assay was conducted with acceptors **87-92** and fungal extract fractions POE-P and S according to the general procedure described in section 5.3c (Table 6.1 and 6.2). A series of no donor control assays were also performed where α-GDP-mannose was replaced with water. Aliquots of all reactions were taken at 30, 60, and 90 min and evaluated by TLC and CE (Table 6.3).

Table 6.1 Concentration and volume of each substrate used in Assay 1.

Substrate	Conc. (mM)	Volume (µL)
87	3.89	2.57
88	2.48	4.03
89	1.86	5.37
90	2.80	3.58
91	1.34	7.49
92	2.36	4.24

Table 6.2 Assay 1 reaction conditions.

	Final Conc. (mM)	Volume (µL)	Volume (µL)
Conditions		A	B
Substrate	0.5	–	–
500mM HEPES, pH 7.0	50.0	2.00	2.00
100mM $MnCl_2$	10.0	2.00	2.00
100mM $MgCl_2$	10.0	2.00	2.00
100mM DTT	10.0	2.00	2.00
H_2O		0.06	0.06
10.31mM α-GDP-Mannose	1.0	1.94	1.94
Fungal Extract Fraction POE-P (0.2%)		10.00	x
Fungal Extract Fraction S		x	10.00
Total Volume		20.00	20.00

Table 6.3 CE results for Assay 1. All CE analysis was performed in TBE buffer, pH 8.28, except those denoted by (*) which were done in TBES (TBE, 20mM SDS), pH 8.28.

Acceptor	Extract Fraction	Product (% conversion)		Time (min)
		1	2	
87	S	72.50	9.62	30
	POE-P	48.52	51.04	30
88	S	28.85	-	30
	POE-P	73.23	-	30
89	S	2.08	-	90
	POE-P	4.64	-	90
90	S	No Reaction		90
	POE-P	No Reaction		90
91	S	CE Inconclusive		30
	POE-P	CE Inconclusive		30
92	S	5.86*	-	90
	POE-P	17.19*	-	90

Assay 2: Detergent Effect on the Conversion of **89** to Product.

Fungal fractions S and P were first solubilized with 0.5% and 1% POE and Triton-X according to the procedure in section 6.3b (Table 6.4). The effect of detergent on acceptor **89** was evaluated with the various fungal extract fractions (Table 6.5). A no donor control assay was performed under assay 2 conditions with α-GDP-mannose substituted with water. Aliquots of the reaction were taken at 2h, 4h, 1 day, 2 days, 3 days, and 7 days and evaluated by TLC and CE (Table 6.6).

Table 6.4 Solubilization conditions of fungal extract fractions S and P used in Assay 2.

			Volume			
Assay	Detergent	Final Det. Conc.	Detergent	Buffer	Fungal Extract S or P	Total
		(%)	(μL)	(μL)	(μL)	(μL)
1	N/A	0		100	500	600
2	10% Triton-X	0.5	30	70	500	600
3	10% Triton-X	1.0	60	40	500	600
4	100% POE	0.5	3.0	97	500	600
5	100% POE	1.0	6.0	94	500	600

Table 6.5 Assay 2 reaction conditions.

	Final Conc. (mM)	Volume (µL)	Volume (µL)	Volume (µL)	Volume (µL)	Volume (µL)
Assay		1	2	3	4	5
1.86 mM Substrate **89**	0.5	5.37	5.37	5.37	5.37	5.37
500mM NH_4OAc, pH 6.0	50.0	2.00	2.00	2.00	2.00	2.00
100mM $MnCl_2$	10.0	2.00	2.00	2.00	2.00	2.00
100mM $MgCl_2$	10.0	2.00	2.00	2.00	2.00	2.00
100mM DTT	10.0	2.00	2.00	2.00	2.00	2.00
H_2O		0.06	0.06	0.06	0.06	0.06
10.31mM α-GDP-Mannose	1.0	1.94	1.94	1.94	1.94	1.94
Fungal Extract Fraction S or P		10.00				
Fungal Extract Fraction S or P 0.5% Triton-X			10.00			
Fungal Extract Fraction S or P 1.0% Triton-X				10.00		
Fungal Extract Fraction S or P 0.5% POE					10.00	
Fungal Extract Fraction S or P 1.0% POE						10.00
Total Volume		20.00	20.00	20.00	20.00	20.00

Table 6.6 CE results for Assay 2.

Acceptor **89** with Fraction P					
	No Detergent	0.5% TX	1.0% TX	0.5% POE	1.0% POE
Time (Hour)	% Conversion	% Conversion	% Conversion	% Conversion	% Conversion
2	0.96	0.74	0.51	1.15	1.27
4	2.48	1.65	1.33	2.16	2.60
24	11.23	8.80	7.84	8.31	11.06
48	20.54	15.72	13.77	11.48	17.85
72	23.51	19.74	17.04	30.03	21.10
168	23.46	24.37	20.70	39.85	24.22

Acceptor **89** with Fraction S					
	No Detergent	0.5% TX	1.0% TX	0.5% POE	1.0% POE
Time (Hour)	% Conversion	% Conversion	% Conversion	% Conversion	% Conversion
2	0.23	1.76	1.72	0.83	2.08
4	0.09	4.48	4.13	2.38	4.68
24	1.11	18.42	16.27	11.09	16.95
48	2.50	22.96	18.31	15.08	20.88
72	2.98	23.28	18.21	16.26	22.03
168	3.22	20.87	16.14	16.21	21.81

Assay 3: Initial Optimization of the Fungal Extract Assay with Acceptor **90**.

Acceptor **90** was screened for mannopyranosyltransferase activity under various assay conditions with fungal extract fraction POE-P for two days (Table 6.7). A no donor control assay was performed under the same conditions as assay 5. Aliquots of the reaction were taken at day 1 and day 2 and evaluated by TLC and CE (Table 6.8).

Table 6.7 Assay 3 Reaction Conditions.

	Final Conc. (mM)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)
Assay		1	2	3	4	5	6	7
2.80mM Substrate **90**	0.5	3.58	3.58	3.58	3.58	3.58	3.58	3.58
500mM NH_4OAc, pH 6.0	50.0	2.00	2.00	2.00	2.00	2.00	x	x
500mM HEPES, pH 7.0	50.0	x	x	x	x	x	2	2.00
500mM Tris, pH 8.0	x	x	x	x	2.00	x	x	x
100mM $MnCl_2$	10.0	2.00	2.00	2.00	2.00	2.00	2.00	2.00
100mM $MgCl_2$	10.0	x	x	x	x	2.00	2.00	2.00
100mM DTT	10.0	2.00	x	x	2.00	2.00	x	x
H_2O		2.06	4.06	2.06	2.06	0.06	2.06	2.06
10.31mM α-GDP-Mannose	1.0	1.94	1.94	1.94	1.94	1.94	1.94	1.94
Fungal Extract Fraction P 0.2% POE		10.00	10.00	10.00	10.00	10.00	10.00	10.00
Total Vol.		20.00	20.00	20.00	20.00	20.00	20.00	20.00

Table 6.8 CE results for Assay 3.

	% Conversion						
Assay	1	2	3	4	5	6	7
Day 1	3.14	3.37	0.21	3.23	4.25	3.38	0.62
Day 2	5.62	6.61	-	-	11.47	7.14	-

Assay 4: Dependence of the Mannopyranosyltransferase Activity on pH with **90.**

The pH dependence of the enzyme was determined in HEPES and ammonium acetate (AA) buffer between pH 4.5 and 7.0 (Table 6.9). No donor control assays were performed under assay 3, 4, and 5 conditions with α-GDP-mannose substituted with water. Aliquots of assays 1-3 and 5 were taken at 1 day, 2 days, 3 days, and 4 days whereas aliquots of assay 4 and 6 were taken at 1 day and 2 days. All samples were evaluated by TLC and CE (Table 6.10).

Table 6.9 Assay 4 Reaction Conditions.

	Final Conc. (mM)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)
Assay		1	2	3	4	5	6
2.80mM Substrate **90**	0.5	3.58	3.58	3.58	3.58	3.58	3.58
500mM NH_4OAc, pH 4.5	50.0	2.00	x	x	x	x	x
500mM NH_4OAc , pH 5.0	50.0	x	2.00	x	x	x	x
500mM NH_4OAc, pH 5.5	50.0	x	x	2.00	x	x	x
500mM NH_4OAc, pH 6.0	50.0	x	x	x	2.00	x	x
500mM NH_4OAc, pH 6.5	50.0	x	x	x	x	2.00	x
500mM HEPES, pH 7.0	50.0	x	x	x	x	x	2.00
100mM MnCl2	10.0	2.00	2.00	2.00	2.00	2.00	2.00
100mM $MgCl_2$	10.0	2.00	2.00	2.00	2.00	2.00	2.00
100mM DTT	10.0	2.00	2.00	2.00	2.00	2.00	x
H_2O		0.06	0.06	0.06	0.06	0.06	2.06
10.31mM α-GDP-Mannose	1.0	1.94	1.94	1.94	1.94	1.94	1.94
Fungal Extract Fraction P 0.2% POE		10.00	10.00	10.00	10.00	10.00	10.00
Total Volume		20.00	20.00	20.00	20.00	20.00	20.00

Table 6.10 CE results for Assay 4.

	% Conversion					
Assay	1	2	3	4	5	6
Day 1	0	0	3.99	4.25	2.94	3.38
Day 2	0	0	9.70	11.47	6.17	7.14
Day 3	0	0	12.04	-	6.70	-
Day 4	0	0	14.95	-	6.96	-

Assay 5: Dependence of the Mannopyranosyltransferase Activity on α-GDP-mannose Concentration with **90**.

The dependence of the reaction on α-GDP-mannose was examined at 1.0M and 2.0M concentrations (Table 6.11). A no donor control assay was performed (Assay 3). Aliquots of assays 1-3 taken after days 1, 2, 4, 7, and 8 were evaluated by TLC and CE (Table 6.12).

Table 6.11 Assay 5 Reaction Conditions.

	Final Conc. (mM)	Volume (μL)	Volume (μL)	Volume (μL)
Assay		1	2	3
6.93mM Substrate **90**	0.5	0.72	0.72	0.72
500mM NH_4OAc, pH 6.0	50.0	1.00	1.50	1.00
100mM $MnCl_2$	10.0	1.00	1.50	1.00
100mM $MgCl_2$	10.0	1.00	1.50	1.00
100mM DTT	10.0	1.00	1.50	1.00
H_2O		0.34	x	1.00
15.109mM α-GDP-Mannose	1.0	0.66	1.99	x
Fungal Extract Fraction P		5.00	7.00	5.00
Total Volume		10.00	10.00	10.00

Table 6.12 CE Results for Assay 5.

	% Conversion	
Assay	1	2
Day 1	4.76	10.64
Day 2	5.91	10.17
Day 4	12.59	18.78
Day 7	14.33	17.74
Day 8	14.41	15.28

Assay 6: Optimization of the Fungal Extract Assay with Acceptor **91**.

Disaccharide **91** was assayed with various solubilization conditions of fungal extract fraction P at pH 5.5, 6.0, and 7.0 (Table 6.13). A no donor control assay was performed under the same conditions as assay 6 with α-GDP-mannose substituted with water. Aliquots were taken at 30, 60, 90 and 1440 min, and were evaluated by TLC and CE (Table 6.14).

Table 6.13 Assay 6 Reaction Conditions.

	Final Conc. (mM)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)	Vol. (µL)
Assay		1	2	3	4	5	6	7	8	9
6.11mM Substrate **91**	0.5	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82
500mM NH_4OAc, pH 5.5	50.0	x	x	x	x	x	x	2.00	2.00	2.00
500mM NH_4OAc, pH 6.0	50.0	x	x	x	x	x	x	x	x	x
500mM HEPES, pH 7.0	50.0	x	x	x	x	x	x	x	x	x
100mM $MnCl_2$	10.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
100mM $MgCl_2$	10.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
100mM DTT	10.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
H2O		0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82
9.26mM α-GDP-Mannose	1.0	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08
Fungal Extract Fraction P		5.00			5.00			5.00		
Fungal Extract Fraction P 0.5% Triton-X			5.00			5.00			5.00	
Fungal Extract Fraction P 0.5% POE				5.00			5.00			5.00
Total Volume		10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00

Table 6.14 CE Results for Assay 6.

Product 1									
	pH 5.5			pH 6.0			pH 7.0		
Time	0.5% POE	0.5% TX	NO DET	0.5% POE	0.5% TX	NO DET	0.5% POE	0.5% TX	NO DET
30	42.08	20.38	23.28	45.38	25.85	21.84	25.43	17.11	22.19
60	63.78	43.04	40.23	56.94	40.34	32.53	25.42	23.77	28.02
90	66.60	49.92	42.11	58.08	48.26	40.42	25.89	27.35	33.49
1440	48.07	49.20	50.44	42.92	35.81	51.14	29.08	25.80	44.55

Product 2									
	pH 5.5			pH 6.0			pH 7.0		
Time	0.5% POE	0.5% TX	NO DET	0.5% POE	0.5% TX	NO DET	0.5% POE	0.5% TX	NO DET
30	12.50	10.31	12.61	17.61	13.97	11.70	49.31	27.36	20.66
60	17.12	17.02	25.71	27.19	24.53	23.23	61.22	42.37	40.71
90	24.16	23.61	36.62	33.89	32.80	31.93	64.79	53.10	46.29
1440	50.76	45.66	43.85	54.69	60.02	35.84	65.43	66.58	49.52

Assay 7: Large Scale Assays with **89** to form 14-*N*-[3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]-7,14-di-aza-8,13-di-oxo-11-thia-tetradecyl α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→2)-β-D-mannopyranoside (**93**).

Monosaccharide acceptor **89** was incubated with non-solubilized fungal extract fraction P for eight days (Table 6.15). One equivalent of α-GDP-mannose was added on day 0 and day 1. Half an equivalent was added every day on days 2 to 8. Aliquots were on days 1 to 5, and 8 and were evaluated by TLC and CE (Table 6.16). The reaction was diluted to 10mL with milli-Q H_2O on day 8. The mixture was initially purified on two sequential C_{18} SepPak cartridges with water and methanol. The solution was concentrated to dryness under reduced pressure. The residue was purified by

reverse phase FPLC with 20mM NH_4OAc, pH 6.0 and MeCN as eluant to afford trisaccharide **93** (46.16% conversion, 85% recovery, 0.563mg). NMR data for Product **93** is found in Table 6.17 and in the subsequent paragraph.

Table 6.15 Assay 7 Reaction Conditions.

	Final Conc. (mM)	Volume (μL)
6.23mM Substrate **89**	0.5	212
500mM NH_4OAc, pH 6.0	50.0	265
100mM $MnCl_2$	10.0	265
100mM $MgCl_2$	10.0	265
100mM DTT	10.0	265
H_2O		620
15.109mM α-GDP-Mannose	1.0	175
Fungal Extract Fraction P		795
Total Volume		2650

Table 6.16 CE Results for Assay 7.

	% Conversion					
Time	Day 1	Day 2	Day 3	Day 4	Day 5	Day 8
Trisaccharide **93**	15.99	33.15	40.99	44.17	45.64	46.16
Oxidized Trisaccharide	0.69	1.59	2.37	2.94	2.81	2.85
Tetrasaccharide	1.11	2.53	3.64	4.12	4.08	4.07
Pentasaccharide	0.16	0.64	1.25	1.45	1.62	1.86

Table 6.17 ^{1}H and ^{13}C NMR Chemical Shifts of **93** obtained from a GHSQC at 600 MHz in CD_3OD.

NMR Assignment of **93**			
Assignment	^{1}H (ppm)	Assignment	^{13}C (ppm)
H-1	4.47	C-1	100.20
H-2	3.98	C-2	75.47
H-3	3.53	C-3	74.66
H-4	3.54	C-4	67.50
H-5	3.18	C-5	77.23
H-6a	3.74		
H-6b	3.71	C-6	61.21
H-1'	5.39	C-1'	99.70
H-2'	4.00	C-2'	78.07
H-3'	3.89	C-3'	70.69
H-4'	3.72	C-4'	67.10
H-5'	4.01	C-5'	72.32
H-6a'	3.84		
H-6b'	3.67	C-6'	61.58
H-1"	4.98	C-1"	102.39
H-2"	3.94	C-2"	70.51
H-3"	3.69	C-3"	71.02
H-4"	3.58	C-4"	67.21
H-5"	3.66	C-5"	73.38
H-6a"	3.84		
H-6b"	3.67	C-6"	61.58

^{1}H NMR (600 MHz, CD_3OD): δ_H, 8.21 (d, 1H, J = 2.0 Hz, aromatic), 7.96 (dd, 1H, J = 2.1, 8.2 Hz, aromatic), 7.19 (d, 1H, J = 8.3 Hz, aromatic), 6.99 (d, 2H, J = 9.0 Hz, aromatic), 6.67 (d, 2H, J = 2.3 Hz, aromatic), 6.63 (d, 1H, J = 2.0 Hz, aromatic), 6.61 (d, 1H, J = 2.0 Hz, aromatic), 5.41 (d, 1H, J = 1.3 Hz, H-1'), 5.00 (d, 1H, J = 1.6 Hz, H-1''), 4.49 (1H, d, J = 0.7 Hz, H-1), 4.05-3.99

(m, 3H, H-2, H-2', H-5'), 3.96 (dd, 1H, J = 1.7, 3.3 Hz, H-2''), 3.91 (dd, 1H, J = 3.3, 9.6 Hz, H-3'), 3.89-3.82 (m, 3H, H-6a', H-6a'', H-A1), 3.79-3.63 (m, 7H, H-3'', H-4', H-5'', H-6a', H-6b, H-6b', H-6b''), 3.60 (dd, 1H, J = 9.5, 9.5 Hz, H-4''), 3.57-3.54 (m, 2H, H-3, H-4), 3.46 (ddd, 1H, J = 6.5, 6.5, 13.0 Hz, H-A2), 3.43 (S, 2H, H-L1, H-L2), 3.22-3.16 (m, 3H, H-5, H-F1, H-F2), 2.97 (t, 2H, J = 7.3 Hz, H-J1, H-J2), 2.57 (t, 2H, J = 7.0 Hz, H-I1, H-I2), 1.62-1.48 (m, 4H, H-B1, H-B2, H-E1,H-E2), 1.41-1.27 (m, 4H, H-C1, H-C2, H-D1, H-D2).

Assay 8: Large Scale Assays with **90** to form 14-*N*-[3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]-7,14-di-aza-8,13-di-oxo-11-thia-tetradecyl α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→2)-β-D-mannopyranoside (**94**).

Disaccharide acceptor **90** was incubated with non-solubilized fungal extract fraction P for three days (Table 6.18). α-GDP-Mannose (1.7 equivalents) were added on day 0 and 1.7 equivalents was added subsequently at 24 and 48 h. Aliquots were taken at 24, 48, and 66 h and were evaluated by TLC and CE (Table 6.19). The reaction was diluted to 15mL with milli-Q H_2O on day 3. The mixture was initially purified on two sequential C_{18} SepPak cartridges with water and MeCN. The solution was concentrated to dryness under reduced pressure. The residue was purified by reverse phase FPLC with 10mM NH_4OAc, pH 5.0 and MeCN as eluant to afford trisaccharide **94** (no yield available). NMR data for Product **94** is found in Table 6.20 and in the subsequent paragraph.

Table 6.18 Assay 8 reaction conditions.

	Final Conc. (mM)	Volume (μL)
X mM Substrate **90***	0.6	x
500mM NH_4OAc, pH 6.0	50.0	300
100mM $MnCl_2$	10.0	300
100mM $MgCl_2$	10.0	300
100mM DTT	10.0	30
H2O		1000
100mM α-GDP-Mannose	1.0	30
Fungal Extract Fraction P		1000
Total Volume		2960

* This assay was performed by a collaborator.

Table 6.19 CE results for Assay 8.

Conversion of **90** to **94**			
Time (H)	16	48	66
% Conversion	15.99	33.15	40.99

Table 6.20 ^{1}H and ^{13}C NMR Chemical shifts of **94** obtained from a GHSQC at 800 MHz in CD_3OD.

NMR Assignment of **94**			
Assignment	^{1}H (ppm)	Assignment	^{13}C (ppm)
H-1	4.48	C-1	102.83
H-2	4.00	C-2	78.15
H-3	3.56	C-3	77.58
H-4	3.55	C-4	70.02
H-5	3.20	C-5	79.89
H-6a	3.75		
H-6b	3.72	C-6	63.58
H-1'	5.40	C-1'	102.76
H-2'	4.01	C-2'	81.01
H-3'	3.90	C-3'	73.32
H-4'	3.73	C-4'	69.94
H-5'	4.02	C-5'	75.17
H-6a'	3.85		
H-6b'	3.68	C-6'	64.52
H-1"	5.00	C-1"	105.04
H-2"	3.95	C-2"	73.25
H-3"	3.71	C-3"	73.38
H-4"	3.60	C-4"	70.00
H-5"	3.68	C-5"	76.16
H-6a"	3.85		
H-6b"	3.68	C-6"	64.52

^{1}H NMR (800 MHz, CD_3OD): δ_H, 8.23 (d, 1H, J = 2.0 Hz, aromatic), 7.94 (dd, 1H, J = 2.1, 8.2 Hz, aromatic), 7.18 (d, 1H, J = 8.3 Hz, aromatic), 6.94 (d, 2H, J = 9.0 Hz, aromatic), 6.67 (d, 2H, J = 2.3 Hz, aromatic), 6.62-6.59 (m, 2H, aromatic), 5.41 (d, 1H, $J \approx 1$ Hz, H-1'), 5.00 (d, 1H, J = 1.6 Hz, H-1''), 4.49 (1H, d, $J \leq 1$ Hz, H-1), 4.04-3.98 (m, 3H, H-2, H-2', H-5'), 3.95 (dd, 1H,

J = 1.7, 5.3 Hz, H-2''), 3.90 (dd, 1H, J = 3.4, 9.7 Hz, H-3'), 3.89-3.82 (m, 3H, H-6a', H-6a'', H-A1), 3.78-3.65 (m, 7H, H-3'', H-4', H-5'', H-6a', H-6b, H-6b', H-6b''), 3.60 (dd, 1H, J = 9.5, 9.5 Hz, H-4''), 3.56-3.53 (m, 2H, H-3, H-4), 3.46 (ddd, 1H, J = 6.6, 6.6, 13.2 Hz, H-A2), 3.42 (S, 2H, H-L1, H-L2), 3.21-3.16 (m, 3H, H-5, H-F1, H-F2), 2.96 (t, 2H, J = 7.1 Hz, H-J1, H-J2), 2.56 (t, 2H, J = 7.0 Hz, H-I1, H-I2), 1.63-1.47 (m, 4H, H-B1, H-B2, H-E1,H-E2), 1.42-1.31 (m, 4H, H-C1, H-C2, H-D1, H-D2).

Assay 9: Large Scale Assays with **91** to form 14-*N*-[3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]-7,14-di-aza-8,13-di-oxo-11-thia-tetradecyl (α-D-mannopyranosyl)-(1→2)-(α-D-mannopyranosyl)-(1→6)-[α-D-mannopyranosyl-(1→3)]-α-D-mannopyranoside (**96**)

Disaccharide acceptor **91** was incubated with non-solubilized fungal extract fraction P for 48 h (Table 6.21). One equivalent of α-GDP-mannose was initially added to the reaction. A second equivalent was supplemented at 18 h. Aliquots were taken at 24 and 48 h and were evaluated by TLC and CE (Table 6.22). The reaction was diluted to 10mL with milli-Q H_2O after 48 h. The mixture was initially purified on two sequential C_{18} SepPak cartridges with water and methanol. The solution was concentrated to dryness under reduced pressure. The residue was purified by reverse phase FPLC with water and methanol containing 0.1% AcOH, 20mM NH_4OAc, pH 6.0 and MeCN, as eluant to afford trisaccharide **95** (25.81% conversion, 84% recovery, 0.253mg) and tetrasaccharide **96** (39.08% conversion, 38% recovery, 0.199mg).

Table 6.21 Assay 9 Reaction Conditions.

	Final Conc. (mM)	Volume (μL)
6.95mM Substrate **91**	0.5	165
500mM NH_4OAc pH 6.0	50.0	204
100mM $MnCl_2$	10.0	204
100mM $MgCl_2$	10.0	204
100mM DTT	10.0	204
H_2O		478
15.11mM α-GDP-Mannose	1.0	135
Fungal Extract Fraction P		613
Total Volume		2042

Table 6.22 CE results for Assay 9.

	% Conversion							
Time (H)	**95**	Oxidized **95**	**96**	Prod 4	Prod 5	Prod 6	Prod 7	Prod 8
24	39.19	8.34	40.26	1.57	1.66	1.88	0.00	0.00
48	25.81	21.04	39.08	3.11	2.51	4.39	0.48	0.11

Table 6.23 ^{1}H and ^{13}C NMR Chemical Shifts of **96** obtained from a GHSQC at 600 MHz in CD_3OD.

NMR Assignment of **96**			
Assignment	1H	Assignment	13C
H-1	4.68	C-1	100.38
H-2	4.01	C-2	70.11
H-3	3.78	C-3	79.48
H-4	3.79	C-4	66.13
H-5	3.66	C-5	72.14
H-6a	3.90		
H-6b	3.69	C-6	66.08
H-1'	5.12	C-1'	98.49
H-2'	3.90	C-2'	79.10
H-3'	3.70	C-3'	71.01
H-4'	3.61	C-4'	67.57
H-5'	3.61	C-5'	72.99
H-6a'	3.82		
H-6b'	3.68	C-6'	61.60
H-1"	4.97	C-1"	102.7
H-2"	3.96	C-2"	70.63
H-3"	3.85	C-3"	70.77
H-4"	3.59	C-4"	67.45
H-5"	3.72	C-5"	73.54
H-6a"	3.82		
H-6b"	3.68	C-6"	61.60
Double and Triple Prime are Interchangeable			
H-1'''	5.06	C-1'''	102.55
H-2'''	3.96	C-2'''	70.63
H-3'''	3.79	C-3'''	71.07
H-4'''	3.61	C-4'''	67.57
H-5'''	3.77	C-5'''	73.51
H-6a'''	3.82		
H-6b'''	3.68	C-6'''	61.60

^{1}H NMR (600 MHz, CD_3OD): δ_H, 8.26 (d, 1H, $J \leq 1$ Hz, aromatic), 7.94 (dd, 1H, $J \leq 1$ Hz, $J = 8.1$ Hz, aromatic), 7.18 (d, 1H, $J = 8.5$ Hz, aromatic), 6.84 (broad d, 2H, aromatic), 6.68 (d, 2H, $J \leq 1$ Hz, aromatic), 6.63-6.53 (m, 2H, aromatic), 5.13 (d, 1H, $J \approx 1.6$ Hz, H-1'), 5.06 (d, 1H, $J = 1.6$ Hz, H-1'''), 4.98 (d, 1H, $J = 1.6$ Hz, H-1''), 4.69 (1H, d, $J = 1.6$ Hz, H-1), 4.01 (dd, 1H, $J = 2.4$, 2.4 Hz, H-2), 3.98-3.95 (m, 2H, H-2'', H-2'''), 3.93-3.88 (m, 2H, H6a, H-2'), 3.89-3.77 (m, 9H, H-3, H-4, H-6a', H-6b', H-3'', H-6a'', H-3''', H-5''', H-6a'''), 3.75-3.55 (m, 12H, H-5, H-6b, H-3', H-4', H-5', H-6b', H-4'', H-5'', H-6b'' H-4''', H-6b''', H-A2), 3.44-3.38 (m, 2H, H-A2, H-N1, H-N2), 3.36 (t, 1H, $J = 10.5$ Hz, H-H1, H-H2), 2.96 (t, 2H, $J = 7.3$ Hz, H-L1, H-L2), 2.63 (t, 2H, $J = 7.3$ Hz, H-G1, H-G2), 2.58 (t, 2H, $J = 6.9$ Hz, H-K1, H-K2), 2.55 (t, 2H, $J = 7.3$ Hz, H-E1, H-E2), 1.65-1.43 (m, 4H, H-B1, H-B2, H-D1, H-D2), 1.36-1.24 (m, 2H, H-C1, H-C2).

Assay 10: α- and β-Mannosidase Assays.

Products from the large scale assays (Assays 7-9, Table 6.24) were incubated with α- and β-mannosidase (Table 6.25). Aliquots were taken at 15 min, 60 min, and overnight and were evaluated by TLC (Table 6.24).

Table 6.24 Assay 10 Substrates and Results.

		Digestion	
Large Scale Reaction Assay	Large Scale Reaction Assay Product	α	β
Substrate **89**	**93**	Y	N
	Tetrasaccharide	Y	N
	Pentasaccharide	Y	N
Substrate **90**	**94**	Y	N
Substrate **91**	**95**	Y	N
	96	Y	N

Table 6.25 Assay 10 Reaction Conditions.

	Final Conc. (mM)	Volume (μL)	Volume (μL)	Volume (μL)
Conditions		A	NE	B
Substrate	–	–	–	–
500mM NH_4OAc, pH 6.0	50.0	1.00	x	x
500mM HEPES, pH 7.0	50.0	x	1.00	1.00
10X BSA	1.0	1.00	1.00	1.00
H_2O		6.00	8.00	6.00
α-Mannosidase	40	2.00	x	x
β-Mannosidase		x	x	2.00
Total Volume		10.00	10.00	10.00

Assay 11: CaMNT1p with acceptors **89**, **90**, and **91**.

Acceptors **89**, **90**, and **91** were incubated with CaMNT1p under the specified reaction conditions (Table 6.26). No donor controls were performed with all substrates. Aliquots were taken at 15 min, 60 min, and overnight and were evaluated by TLC (Table 6.24). Analysis of selected aliquots by CE and the associated results are outlined in Table 6.27

Table 6.26 Assay 11 Reaction Conditions.

	Final Conc. (mM)	Volume (μL)	Volume (μL)
Conditions		A	B
Substrate	0.5	–	–
500mM NaOAc pH 6.0	50.0	1.00	1.00
100mM $MnCl_2$	10.0	1.00	1.00
100mM DTT	10.0	1.00	1.00
H2O		1.30	2.00
14.23mM α-GDP-Mannose	1.0	0.70	x
Crude MNT1		5.00	5.00
Total Volume		10.00	10.00

Table 6.27 CE Results for Assay 11.

		% Conversion	
	Product	60 Minutes	Overnight
89	Trisaccharide	22.07	75.08
	Oxidized Trisaccharide	1.32	5.32
	Tetrasaccharide	0.00	5.82
90	Trisaccharide	–	0.35
	Unknown	–	0.11
91	Trisaccharide	–	11.89
	Oxidized Trisaccharide	–	2.34
	Tetrasaccharide	–	3.08
	Unknown	–	3.94
	Unknown	–	0.71
	Unknown	–	1.14
	Unknown	–	0.49

CHAPTER 7

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